



PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵: A01K 67/00, A61K 49/00, C12N 5/00, 15/00, C12Q 1/00, G01N 31/00, 33/48, 33/53		A1	(11) International Publication Number: WO 94/26100 (43) International Publication Date: 24 November 1994 (24.11.94)
(21) International Application Number: PCT/US94/05746 (22) International Filing Date: 18 May 1994 (18.05.94) (30) Priority Data: 08/062,850 18 May 1993 (18.05.93) US (60) Parent Application or Grant (63) Related by Continuation US 08/062,850 (CIP) Filed on 18 May 1993 (18.05.93) (71) Applicants (for all designated States except US): INSTITUTE NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE [FR/FR]; 101, rue de Tolbiac, F-75654 Paris (FR). CENTRE NATIONAL DE LA RECHERCHE SCI- ENTIFIQUE [FR/FR]; 3, rue Michel-Ange, F-75794 Paris Cédex 16 (FR). UNIVERSITE LOUIS PASTEUR, STRAS- BOURG I [FR/FR]; 4, rue Blaise-Pascal, Boîte postale 1032/F, F-67070 Strasbourg Cédex (FR). E.R. SQUIBBS & SONS, INC. [US/US]; Lawrenceville-Princeton Road, Princeton Road, Princeton, NJ 08543-4000 (US).		(71)(72) Applicant and Inventor: MENDELSON, Cathy [US/US]; 7b East Hamilton Avenue, Englewood, NJ 07631 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): CHAMBON, Pierre [FR/FR]; 4, rue du Dr.-Albert-Schweitzer, F-67113 Blaesheim (FR). LUFKIN, Thomas [US/US]; Apartment 16C, 166 East 63rd, New York, NY 10029 (US). LOHNES, David [CA/FR]; 7C, rue des Lentilles, F-67000 Strasbourg (FR). MARK, Manuel [FR/FR]; 8, rue du Château-d'Eau, F-67350 Marschwiller (FR). DIERICH, Andrée [FR/FR]; 60, route du Polygone, F-67100 Strasbourg (FR). GORRY, Philippe [FR/FR]; 10, rue de Mulhouse, F-67100 Strasbourg (FR). KASTNER, Philippe [FR/FR]; 1, rue du Tonnelet- Rouge, F-67000 Strasbourg (FR). LEMEUR, Marianne [FR/FR]; 14, rue Jean-Jacques-Waltz, F-67200 Strasbourg (FR). (74) Agents: GOLDSTEIN, Jorge, A. et al.; Sterne, Kessler, Goldstein & Fox, Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US). (81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>	
(54) Title: GENETICALLY ENGINEERED MICE CONTAINING ALTERATIONS IN THE GENES ENCODING RETINOIC ACID RECEPTOR PROTEINS (57) Abstract <p>The present invention provides mice which are deficient in the normal expression of one or more members of the RAR or RXR class of receptors, to mice heterozygous for such deficiency, and to cell lines, preferably pluripotent or totipotent cell lines, which are heterozygous or homozygous for such deficiency. The present invention further provides the use of any of the above mice and cell lines in situations where the absence of at least one RAR or RXR receptors, or the normal expression thereof, is desirable.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LU	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TG	Togo
CZ	Czech Republic	MD	Republic of Moldova	TJ	Tajikistan
DE	Germany	MG	Madagascar	TT	Trinidad and Tobago
DK	Denmark	ML	Mali	UA	Ukraine
ES	Spain	MN	Mongolia	US	United States of America
FI	Finland			UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

- 1 -

Genetically Engineered Mice Containing Alterations In The Genes Encoding Retinoic Acid Receptor Proteins

Cross Reference to Related Application

5 This application is a continuation-in-part of U.S. patent application
08/062,850, filed May 18, 1993, the contents of which are incorporated herein
by reference.

Field Of The Invention

10 The present invention relates to the fields of retinoic acid receptor
(RAR) biology and transgenic mice. Specifically, the present invention relates
to mice which are deficient in the normal expression of one or more of the
genes encoding members of the RAR or RXR class of receptors, to mice
heterozygous for such deficiency, to cell lines, preferably pluripotent or
totipotent cell lines, which are heterozygous or homozygous for such
deficiency, and to methods of using said mice or said cell lines to identify
15 agonists and antagonists of specific members of the RAR or RXR class of
receptors.

Description Of The Related Art

It has long been established that retinoids (vitamin A derivatives) are
crucial for normal growth, vision, maintenance of numerous tissues,

- 2 -

reproduction and overall survival (Wolbach, S.B., and Howe, P.R., *J. Exp. Med.* 42:753-777 (1925); for reviews and refs see Sporn *et al.*, *The retinoids*, Vols. 1 and 2, Sporn *et al.*, eds., Academic Press, Orlando, Florida (1984); Livrea and Packer, in *Retinoids*, Livrea and Packer, eds., Marcel Dekker, New York (1993)). In addition offspring of vitamin A deficient (VAD) dams exhibit a number of developmental defects, indicating that retinoids are also important during embryogenesis (Wilson, J.G., *et al.*, *Am. J. Anat.* 92:189-217 (1953)). With the exceptions of vision (Wald, 1968) and possibly of spermatogenesis in mammals (Thompson *et al.*, *Proc. Royal Soc.* 159:510-535 (1964); van Pelt, H.M.M., and De Rooij, D.G., *Endocrinology* 128:697-704 (1991); and refs therein), most of the effects generated by VAD in fetuses, young, and adult animals can be prevented and/or reversed by retinoic acid (RA) administration (Wilson, J.G., *et al.*, *Am. J. Anat.* 92:189-217 (1953); Thompson *et al.*, *Proc. Royal Soc.* 159:510-535 (1964)). The dramatic teratogenic effects of maternal RA administration on mammalian embryos (Shenefelt, R.E., *Teratology* 5, 103-108 (1972); Lammer *et al.*, *N. Eng. J. Med.* 313:837-841 (1985); Webster, W.S. *et al.*, *J. Cranofac. Genet. Dev. Biol.* 6:211-222 (1986); Kessel and Gruss, *Cell* 67:89-104 (1991); Kessel, M., *Development* 115:487-501 (1992); Creech Kraft, J., "Pharmacokinetics, placental transfer, and teratogenicity of 13-cis-retinoic acid, its isomer and metabolites," In *Retinoids in Normal Development and Teratogenesis*, G.M. Morriss-Kay, ed., Oxford University Press, pp. 267-280 (1992)), and the spectacular effects of topical administration of retinoids on embryonic development of vertebrates and limb regeneration in amphibians (Mohanty-Hejmadi *et al.*, *Nature* 355:352-353 (1992); for review and refs see Tabin, C.J., *Cell* 66:199-217 (1991)), has markedly contributed to the belief that RA could in fact be a morphogen (conferring positional information during development), and may also play a critical role during organogenesis.

With the exception of visual perception (Wald, G. *et al.*, *Science* 162:230-239 (1968)), the molecular mechanisms underlying the highly diverse effects of retinoids has remained obscure until recently. The discovery of nuclear receptors for RA (Petkovich *et al.*, *Nature* 330:444-450 (1987);

Giguère *et al.*, *Nature* 330:624-629 (1987)) has greatly advanced the understanding of how these simple molecules could exert their pleiotropic effects (for reviews see Leid *et al.*, *TIBS* 17:427-433 (1992); Linney, E., *Current Topics in Dev. Biol.* 27:309-350 (1992)). It is thought that the effects of the RA signal are mediated through two families of receptors which belong to the superfamily of ligand-inducible transcriptional regulatory factors that include steroid/thyroid hormone and vitamin D3 receptors (for reviews see Evans, R.M., *Science* 240:889-895 (1988); Green and Chambon, *Trends Genet.* 4:309-314 (1988); Beato, M., *Cell* 56:335-344 (1989); Gronemeyer, H., *Ann. Rev. Genet.* 25:89-123 (1991); de Luca, L.M., *FASEB J.* 5:2924-2933 (1991); Linney, E., *Current Topics in Dev. Biol.* 27:309-350 (1992); Yu, V.C. *et al.*, *Cur. Op. Biotech.* 3:597-602 (1992); Leid *et al.*, *TIBS* 17:427-433 (1992)).

The RAR family (RAR α , β and γ and their isoforms) are activated by both all-trans and 9-cis RA, whereas the retinoid X receptor family (RXR α , β and γ) are activated exclusively by 9-cis RA (for review and refs see de Luca, L.M., *FASEB J.* 5:2924-2933 (1991); Linney, E., *Current Topics in Dev. Biol.* 27:309-350 (1992); Yu, V.C. *et al.*, *Cur. Op. Biotech.* 3:597-602 (1992); Leid *et al.*, *TIBS* 17:427-433 (1992); Kastner *et al.*, "The role of nuclear retinoic acid receptors in the regulation of gene expression," in *Vitamin A in health and disease*, R. Blomhoff, ed., Marcel Dekker, New York (1993); Allenby *et al.*, *Proc. Natl. Acad. Sci. USA* 90:30-34 (1993)). Within a given species, the DNA binding (region C) and the ligand binding (region E) domains of the three RAR types are highly similar, whereas the C-terminal region F and the middle region D exhibit no or little similarity. The amino acid sequences of the three RAR types are also notably different in their B regions, and their main isoforms (α 1 and α 2, β 1 to β 4, and γ 1 and γ 2) further differ in their N-terminal A regions (reviewed in Leid *et al.*, *TIBS* 17:427-433 (1992)). Similarly, the RXRs characterized to date also markedly differ in their N-terminal A/B regions (Leid *et al.*, *TIBS* 17:427-433 (1992); Leid *et al.*, *Cell* 68:377-395 (1992); Mangelsdorf *et al.*, *Genes and Dev.* 6:329-344 (1992)). Amino acid sequence comparisons revealed that the interspecies

conservation of a given RAR or RXR type is greater than the similarity found between the three RAR or RXR types within a given species (reviewed in Leid *et al.*, *TIBS* 17:427-433 (1992)). This interspecies conservation is particularly striking in the N-terminal A regions of the various RAR α , β and γ isoforms, whose A region amino acid sequences are very divergent from each other. Taken together with the distinct spatio-temporal expression patterns observed for the transcripts of each RAR and RXR type in the developing embryo and various adult mouse tissues (Zelent, A., *et al.*, *Nature* 339:714-717 (1989); Dollé *et al.*, *Nature* 342:702-705 (1989); Dollé *et al.*, *Development* 110:1133-1151 (1990); Ruberte *et al.*, *Development* 108:213-222 (1990); Ruberte *et al.*, *Development* 111:45-60 (1991); Mangelsdorf *et al.*, *Genes and Dev.* 6:329-344 (1992)) this interspecies conservation has suggested that each RAR and RXR type (and isoform) may perform unique functions. This hypothesis is further supported by the finding that the various RAR isoforms and RXR types contain two transcriptional activation functions (AFs) located in the N-terminal A/B region (AF-1) and in the C-terminal E region (AF-2), which can synergistically, and to some extent differentially, activate various RA-responsive promoters (Leid *et al.*, *TIBS* 17:427-433 (1992); Nagpal *et al.*, *Cell* 70:1007-1019 (1992)). Moreover, it has been shown that activation of RA-responsive promoters likely occurs through RAR:RXR heterodimers rather than through homodimers (Yu, V.C. *et al.*, *Cell* 67:1251-1266 (1991); Leid *et al.*, *Cell* 68:377-395 (1992b); Durand *et al.*, *Cell* 71:73-85 (1992); Nagpal *et al.*, *Cell* 70:1007-1019 (1992); Zhang, X.K., *et al.*, *Nature* 355, 441-446 (1992); Kliewer *et al.*, *Nature* 355:446-449 (1992); Bugge *et al.*, *EMBO J.* 11:1409-1418 (1992); Marks *et al.*, *EMBO J.* 11:1419-1435 (1992); for reviews see Yu, V.C. *et al.*, *Cur. Op. Biotech.* 3:597-602 (1992); Leid *et al.*, *TIBS* 17:427-433 (1992); Laudet and Stehelin, *Curr. Biol.* 2:293-295 (1992); Green, S., *Nature* 361:590-591 (1993)). Thus, the basis for the highly pleiotropic effect of retinoids may reside, at least in part, through the control of different subsets of retinoid-responsive promoters by cell-specifically expressed heterodimeric combinations of RAR:RXR types (and isoforms),

- 5 -

whose activity may be regulated by cell-specific levels of all-trans and 9-cis RA (Leid *et al.*, *TIBS* 17:427-433 (1992)).

The apparently ubiquitous distribution of RAR α transcripts (mainly the RAR α 1 isoform; Zelent, A., *et al.*, *Nature* 339:714-717 (1989); Leroy *et al.*, *EMBO J.* 10:59-69 (1991); Leroy *et al.*, *Proc. Natl. Acad. Sci. USA* 88:10138-10142 (1991); Dollé *et al.*, *Nature* 342:702-705 (1989); Dollé *et al.*, *Development* 110:1133-1151 (1990); Ruberte *et al.*, *Development* 111:45-60 (1991); E. Ruberte, P. Dollé, D. Decimo and P.C., unpublished results) during development and in adult tissues suggests that RAR α 1 may play some general housekeeping function (Brand *et al.*, *Nucl. Acid Res.* 18:6799-6806 (1990); Leroy *et al.*, *EMBO J.* 10:59-69 (1991)). RAR β transcripts exhibit a more restricted pattern of distribution in developing embryos and adult tissues, suggesting that RAR β isoforms could be involved in the differentiation of certain epithelia, as well as in the ontogenesis of the nervous system (Dollé *et al.*, *Development* 110:1133-1151 (1990); Ruberte *et al.*, *Development* 111:45-60 (1991); Mendelsohn *et al.*, *Development* 113:723-734 (1991)). *In situ* hybridization studies indicate that RAR γ transcripts are apparently restricted to the presomitic caudal region of day 8.0 p.c. embryos, and to the frontonasal mesenchyme, pharyngeal arches, sclerotomies and limb bud mesenchyme at day 8.5 to 11.5 p.c. At later stages, RAR γ transcripts are found in precartilaginous condensations (day 12.5 p.c.), with subsequent restriction to cartilage and differentiating squamous keratinizing epithelia (day 13.5 p.c.), regardless of their embryonic origin (Dollé *et al.*, *Nature* 342:702-705 (1989); Dollé *et al.*, *Development* 110:1133-1151 (1990); Ruberte *et al.*, *Development* 108:213-222 (1990)). These observations suggest a role for RAR γ in morphogenesis, chondrogenesis and differentiation of squamous epithelia (Dollé *et al.*, *Development* 110:1133-1151 (1990); Ruberte *et al.*, *Development* 108:213-222 (1990)). In addition, Northern blot analysis indicates that the RAR γ 2 isoform is the predominant isoform in the early embryo (day 8.5 to 9.5 p.c.), whereas RAR γ 1 is the predominant RAR γ isoform transcript found later in embryogenesis as well as in newborn and adult skin (Kastner *et al.*, *Proc. Natl. Acad. Sci. USA* 87:2700-2704 (1990)).

- 6 -

The mouse is the model of preference in the study of the mammalian genetic system, and a great deal of research has been performed to map the murine genome.

5 It would be of great importance to be able to establish a living model wherein the role of the various members of the RAR class of receptors could be studied in a definitive manner.

10 Accordingly, it is an object of the present invention to generate strains of mice which do not express, or express at undetectable levels, one or more members of the RAR or RXR class of receptors. Such mice would be of great value for a better understanding of the role each of the members of the RAR or RXR class of receptors because such animals and cell lines would allow direct testing of the function of specific genes, either deleted or reintroduced by transgenesis, and would serve as an assay system to identify compounds which act as antagonists or agonists of specific members of the RAR or RXR class of receptors.

15

Summary Of The Invention

To establish the actual functional role of RAR and RXR isoforms *in vivo* during mouse development and post-natal life, the present invention describes the generation of transgenic mice, produced via homologous recombination in embryonic stem (ES) cells, in which RAR γ 2, all RAR γ isoforms, RAR α 1, all RAR α isoforms, RAR β 2, or all RXR α isoforms have been functionally inactivated.

20

Thus, the present invention provides mice and mouse cell lines which are deficient in the normal expression (either incapable of total or detectable functional expression) of one or more subtypes or specific isoforms of RAR or RXR receptors. Specifically, the present invention describes mice and cell lines which have been genetically altered such that the normal expression of one or more of the genes encoding a subtype or specific isoform of a RAR or RXR receptor has been disrupted such that it no longer encodes functional or detectable levels of the given receptor subtype or isoform.

25

30

- 7 -

The invention further provides mice and cell lines which are heterozygous for the above deficiency.

Utilizing one or more of the aforementioned mice or cell lines, the present invention further provides methods of identifying antagonists and agonists of specific members of the RAR or RXR class of receptors. Specifically, the isoform or subtype of RAR or RXR specific for a given agent, and the effects the agent has on inducing RA dependent expression, can be assayed by first incubating an agent with a cell line, a transgenic mouse, or cells or tissues derived therefrom, which is deficient in the normal expression of one or more isoforms or subtypes of RAR or RXR receptors, and then determining the amount of agent bound or determining the level of RA dependent gene expression which is induced in the cell lines, or specific tissues of the transgenic mice.

Brief Description Of The Figures

Figure 1 (Panels a-d). Generation of RAR γ mutant mice.

RAR γ locus, RAR γ 2 and RAR γ targeting constructs, targeted loci, heterozygote inbreeding analysis, and RNase protection analysis.

(Panel a) Diagram of the RAR γ locus, targeting constructs and targeted alleles. The RAR γ A to F regions, the DNA-binding domain (DBD) and ligand-binding domain (LBD) are shown at the top, as are the alternate promoters (P1 and P2) and alternate splicing of exons (E1-E8) which generate the major RAR γ isoforms, RAR γ 1 and RAR γ 2. The two targeting constructs are shown below the RAR γ locus. Plasmid vector sequences are not shown, nor are the constructs drawn to scale. The predicted RAR γ 2 and RAR γ targeted alleles, the restriction enzyme digests and the DNA probes (Probes 1 and 2) used for Southern blotting are shown below. The locations of the neomycin resistance and HSV tk genes are denoted by GTI-II.NEO and GTI-II.tk respectively. B, BamHI; E, EcoRI; Ea, EagI; K, KpnI.

(Panel b) Southern blot analysis of offspring from intermatings of RAR γ 2^{+/-} (top) or RAR γ ^{+/-} (bottom) mice. The positions of the wild type

- 8 -

(WT, +) and mutant (-) alleles are shown to the right and the size of each allele is shown to the left of each blot. The genotype is indicated above each lane. +/+, WT; +/-, heterozygote; -/-, homozygote. The probes (Probe 1, RAR γ analysis; Probe 2, RAR γ 2 analysis) and digests (KpnI, RAR γ 2; BamHI, RAR γ) correspond to the probes and restriction digests shown in panel a.

(Panel c) Representation of the strategy for RNase protection analysis of WT and mutated RAR γ transcripts. RAR γ 1 and RAR γ 2 WT transcripts are represented at the top of the figure, with the predicted RAR γ 2 and RAR γ mutated transcripts shown immediately below. NEO indicates the position of the neomycin resistance gene in each transcript resulting from targeting of the respective cognate alleles. The riboprobe used to detect WT and mutated RAR γ transcripts is shown in the middle of the diagram, followed by the protected fragments for both WT and mutated RAR γ 1 and RAR γ 2 RNAs as indicated on the left. The identity and size in nucleotides (nt) for each protected fragment is shown on the right of the figure. mut, protected fragment derived from RNase protection of mutated RAR γ transcripts.

(Panel d) RNase protection analysis of RNA from either day 10.5 p.c. (RAR γ 2) or day 13.5 p.c. (RAR γ) WT, heterozygote and homozygote embryos for either the RAR γ 2 (lanes 1-3) or RAR γ (lanes 4-6) disruption. The identities of the protected fragments are indicated to the right. The size of the RAR γ 2 WT, RAR γ 2 mutant or RAR γ 1 WT fragments are indicated to the left of each gel. The source of the RNA used in the protection assays was as follows; lane 1, RAR γ 2 WT; lane 2, RAR γ 2^{+/+}; lane 3, RAR γ 2^{-/-}; lane 4, RAR γ WT; lane 5, RAR γ ^{+/+}; lane 6, RAR γ ^{-/-}. The Histone H4 protection was included as an internal control for the quantitation and integrity of the RNA samples. The protected fragment for the RAR γ 1 mutated transcript obtained from RAR γ ^{-/-} or RAR γ ^{+/+} samples was not seen on this autoradiogram due to the small size of the fragment (41 nt; see panel c).

Figure 2 (Panels a-l). Axial skeletal and tracheal cartilage defects in RAR γ null fetuses.

- 9 -

(a to d), lateral view of the occipital, cervical and upper thoracic region of WT (a, c) and $RAR\gamma^{-/-}$ (b, d) skeletons. (a and b), the large arrow in panel b between the basioccipital bone (BO) and the anterior arch of the atlas (AAA) indicates an ossified fusion between these structures (see also Fig. 3a to d); the large arrow in panel b between "C1" and the third cervical vertebra indicates a fusion between the neural arches of these vertebrae; AAA*, ectopic anterior arch of atlas; "C1", anterior transformation of the second cervical vertebra to a first cervical identity; "C6", anterior transformation of the seventh cervical vertebra to a sixth cervical identity (see also panels i to l); C1 to C7, first to seventh cervical vertebrae; E, exoccipital bone; T1, first thoracic vertebra; TR, tympanic ring. (c and d), fusion of first and second ribs (large arrow in panel d), fusion and disruption of tracheal cartilaginous rings (T in panel d, compare to T in panel c; see also Fig. 3e and f), and ectopic anterior arch of the atlas (AAA* in panel d, compare to panels a and c). Numbering (1 and 2) indicates the first and second vertebrosteral ribs. (e, f and g), lateral (e and f) and ventral (g) views of WT (e) or $RAR\gamma^{-/-}$ (f and g) skeletons. *8 (panel f) or R8* (panel g) indicates anterior transformation of the eighth thoracic vertebra to a seventh thoracic identity; arrows indicate bilateral fusions between the first and second ribs (panel g, see also panel d). Numbering (1-7 in panels e and f), normal vertebrosteral ribs; R1-R13 (panel g), ribs. (h), the large arrow indicates fusion between the first and second cervical vertebrae (compare to panel a). (i to l), fifth to seventh cervical vertebrae from either a WT (i) or $RAR\gamma^{-/-}$ (j to l) skeletons. (i), normal aspect of fifth, sixth and seventh cervical vertebrae showing normal foramina transversaria (FT) on the fifth and sixth vertebrae, and the normal (bilateral) positioning of the tuberculi anterior (TA) on the sixth vertebra; (j to l), unilateral anterior transformation of the seventh cervical vertebrae to a sixth vertebral identity ("C6"; panels j to l) inferred from the presence of ectopic tuberculi anterior on the seventh cervical vertebrae ("TA"; panels j to l), and either a complete or partial ectopic foramina transversarium ("FT" or large arrow respectively; panel j); unilateral anterior transformation of the sixth cervical vertebra to a fifth cervical identity

- 10 -

("C5"; panels j to l) inferred from the lack of a tuberculum anterior (arrowhead, panels j to l). Limbs were removed from the skeletons to facilitate analysis. All skeletons were derived from day 18.5 p.c. fetuses with the exception of the skeleton shown in panel h, which was from an 8 day p.p. animal.

Figure 3 (Panels a-f). Skeletal defects in $RAR\gamma$ null homozygotes at the cranio-vertebral junction and malformation of the cartilaginous tracheal rings.

(a) and (b) frontal histological sections of WT (a) and $RAR\gamma^{-/-}$ day 18.5 p.c. fetus (b) at the level of the rostral border of the foramen occipital magnum. In the $RAR\gamma^{-/-}$ animal, the basioccipital (BO) is attached to the lateral mass of the first cervical vertebra (C1L) by the transverse ligament of the atlas (TL) due to the persistence of the first hypochordal bar (H1 in panel b; compare with a); C1-C2, fused bodies of the atlas (C1) and axis (C2); C3, third cervical vertebra. (c and d), sagittal median sections in the occipital and upper cervical region of a WT (c) or $RAR\gamma^{-/-}$ (d) day 18.5 p.c. fetus. A persistent first hypochordal bar (H1) joins the basioccipital (BO) to the anterior arch of the atlas (AAA) in the $RAR\gamma^{-/-}$ fetus. D, axis dens. (e and f), sagittal median histological sections through the cervico-thoracic region of a WT (e) or $RAR\gamma^{-/-}$ day 18.5 p.c. fetus (f). Some of the tracheal cartilaginous rings (R) in the $RAR\gamma^{-/-}$ fetus are fused ventrally (large arrow in panel f). A, arytenoid cartilage; C, cricoid cartilage; T, thyroid cartilage; S, sternum; V, vertebrae.

Figure 4 (Panels a-o). Glandular defects in $RAR\gamma$ null homozygotes (a-k), stratified squamous metaplasia of the epithelia of the seminal vesicles and cranial prostates in 3-month-old $RAR\gamma^{-/-}$ mice.

(a), comparison of ventral views of the left seminal vesicle (SV) and of the cranial prostate (CP) of $RAR\gamma^{-/-}$ and WT littermates. The white color of the WT seminal vesicle is due to the secretion products (S in panel d) of the glandular epithelium which accumulate within the lumen of the organ. The

RAR $\gamma^{-/-}$ seminal vesicle is atrophic and does not display the characteristic white color of the functional organ of normal, sexually mature, males. The histology of this specimen is shown in panels h and i. (b and c), view from above (b) and dorsal view (c) of the seminal vesicles (SV) and cranial prostates (CP) of two RAR $\gamma^{-/-}$ mice, dissected with the testes (T), epididymis (E), vas deferens (D) and urinary bladder (U), and photographed at the same magnification. The aspect of the seminal vesicle (SV) and cranial prostate (CP) of RAR $\gamma^{-/-}$ mice varies from atrophic (panel b) to hypertrophic (e.g. the cranial prostate on the right of the picture in panel c). Hypertrophy was always associated with histological signs of inflammation (see panel l). In the specimen shown in panel b, the seminal vesicle and the cranial prostate (SV+CP) are indistinguishable by both anatomical and histological criteria (see panels j and k). (d-g), histological sections of the seminal vesicle (panels d and e) and cranial prostate (panels f and g) of a 3-month-old WT mouse. The wild-type seminal vesicle is an elongated hollow organ (panel d) whereas the wild-type cranial prostate (CP, or coagulating gland) is composed of several tubules (panel f). Both glands display very irregular lumens filled with secretion products (S, panels d-g). Their walls consist of a mucosa, forming multiple folds and septa, and in a peripheral layer of smooth muscle cells (MU, panels e, g). The glandular epithelia (GE, panels e, g) are simple columnar. (h-l), histological sections of the seminal vesicle and cranial prostate of three different RAR $\gamma^{-/-}$ mice; panels h and i, correspond to the RAR $\gamma^{-/-}$ specimen displayed in panel a; in the seminal vesicle (SV), the epithelium is stratified, squamous and nonkeratinized (SQ); in the cranial prostate, foci of normal glandular epithelial cells (GE, enlarged in i) and patches of squamous nonkeratinized metaplasia (SQ) coexist; the lumens of both glands are devoid of secretion products; panels j and k correspond to the specimen displayed in panel b; both glands are replaced by large cysts filled with desquamated keratinized cells (K); the cysts are lined by a stratified squamous keratinizing epithelium (SQK) resembling normal epidermis; panel l shows an histological section of the specimen displayed in panel c; the connective tissue of the seminal vesicle is markedly hyperplastic and infiltrated

- 12 -

with leukocytes (L) which also occupy the spaces between the desquamated keratinized cells (K) within the lumen of the gland. (m-o), atrophy of the Harderian glands in 3-month-old $RAR\gamma^{-/-}$ mice; panel m, dorsal view of the left (L) and right (R) Harderian glands of two different $RAR\gamma^{-/-}$ mice; the right glands display a normal aspect: conical-shaped with medially-directed tips; the base of the gland on the upper left of the photograph is atrophic and colored in black: this aspect reflects a lack of part of the glandular epithelium without concomitant disappearance of the melanocyte-rich connective tissue of the gland (see panel o); the unformed black structure displayed on the lower left of the photograph replaced the left Harderian gland in the orbit of this mouse; panels n and o, frontal sections through the orbits of a $RAR\gamma^{-/-}$ mouse at the level of the left (panel n) and right (panel o) Harderian glands; panel n, the histological structure of the Harderian gland is normal: it consists of tubules lined by a simple cuboidal glandular epithelium (GE), and of an intertubular connective tissue containing numerous melanocytes (M); in panel o, the epithelium of the Harderian gland is absent. An intraorbital accumulation of closely-packed melanocytes indicates the place where the gland should have been located. MU, orbital muscle. x54 (d, f, h, j); x134 (l); x268 (n,o); x540 (e, g, i, k).

Figure 5 (Panels a-h). Retinoic acid-induced skeletal malformations.

(a to d), lateral views of untreated WT (a), day 8.5 p.c. RA-treated $RAR\gamma^{-/-}$ (b), $RAR\gamma^{+/-}$ (c) and $RAR\gamma^{+/+}$ (d) skeletons. C, T, L and S; first cervical, thoracic, lumbar, and sacral vertebrae, respectively. (e to h), higher magnification ventral views of the specimens shown in panels a to d; T14* and T15* in panels f and g indicate additional thoracic vertebrae formed by anterior transformation of the first and second lumbar vertebrae, respectively; L1* in panels f and g indicate anterior transformation of the second lumbar vertebrae to a first lumbar identity; L6* and S1* in panel f denote anterior transformation of the first and second sacral vertebrae to a sixth lumbar and first sacral identity, respectively; the arrow in panel h indicates degenerate ribs; T13, L1, L6 and S1 in panel e, normal position of the last thoracic, first

- 13 -

and last lumbar and first sacral vertebrae, respectively. Limbs, which were present in all cases, were removed to facilitate analysis.

Figure 6 (Panels a-d)

(Panel a) Diagram of the RAR α targeting constructs, wild type RAR α locus, and disrupted alleles. The various regions of the RAR α protein (A-F), the DNA-binding domain (DBD) and ligand-binding domain (LBD) are indicated at the top (Leroy *et al.*, *EMBO J.* 10:59 (1991)). The alternate promoter (P1 or P2) usage and alternate splicing of exons (E1-E8), which generate the $\alpha 1$ and $\alpha 2$ isoforms, are also shown. The two targeting constructs are drawn above the wild type (WT) RAR α locus. The RAR $\alpha 1$ targeting construct (left) has the neomycin resistance gene (NEO) inserted into the A1 region encoded by Exon 3 (E3), and has a HSV-thymidine kinase gene (tk) at its 5' end. The RAR α targeting construct (right; note that it does not include the tk gene) has the neomycin resistance gene (NEO) inserted into the B region which is encoded by Exon 8 (E8).

The plasmid vector sequences are not shown. The structure of the targeted alleles, and the restriction enzyme digests and DNA probes used for Southern blotting, are indicated below. B, BglII; H, HindIII; K, KpnI; N, NotI; R, EcoRI; RV, EcoRV; S, SpeI; Sa, Sall; X, XbaI.

(Panel b) Southern blots of offspring from intermatings of mice heterozygous for either RAR α or RAR $\alpha 1$ disruptions. The positions of the wild type (+) and mutant (-) alleles are indicated, as well as their size. Genotypes of the offspring are indicated below. +/+, wild type; +/-, heterozygote; -/-, homozygote. The probes indicated (PROBE 1 and PROBE 2) correspond to the probes diagrammed in panel a.

(Panel c) RNase protection analysis of RNA from day 13.5 p.c. +/+, +/- and -/- embryos for either the RAR α (lanes 1-4) or RAR $\alpha 1$ (lanes 5-7) disruptions. The identities of the protected fragments (RAR α , RAR β , RAR γ , CRABPI, CRABPII, and Histone H4) are indicated by the arrows. In the case of RAR β and RAR γ only the protected fragments corresponding to the major isoforms RAR $\beta 2$ and RAR $\gamma 1$ are shown; similar results were

- 14 -

obtained for the other isoforms (RAR β 1, β 3 and β 4; RAR γ 2; data not shown). The source of RNAs used in the protection assays was as follows: lane 1, tRNA (negative control); lane 2, RAR α +/-; lane 3, +/+; lane 4, RAR α -/-; lane 5, +/+; lane 6, RAR α 1 +/-; lane 7, RAR α 1 -/-. The Histone H4 RNA protection was included as a control for the integrity and quantitation of the RNA samples.

(Panel d) Western blot analysis of day 13.5 p.c. embryo nuclear proteins isolated from RAR α +/+, +/-, and -/- embryos. Embryos from RAR α heterozygote matings were removed at day 13.5 p.c., the yolk sac taken for DNA genotyping, and each embryo was frozen individually on dry ice and stored at -80°C (Lufkin *et al.*, *Cell* 66:1105 (1991)).

Nuclear protein extracts were derived from: lanes 1 and 2, transfected Cos-1 cells expressing RAR α 1 and RAR α 2, respectively (positive controls); lane 3, RAR α +/+ embryos; lane 4, RAR α -/- embryos; lane 5 and 6, RAR α -/- embryos; lanes 7-10, transfected Cos-1 cells expressing either RAR β 1, β 2, β 3 or β 4 (positive control); lane 11, RAR α +/+ embryos; lane 12, RAR α +/- embryos; lane 13 and 14, RAR α -/- embryos. RAR α -specific and RAR β -specific antisera were used in lanes 1-6 and 7-14, respectively. 1-5 μ g of Cos-1 transfected protein extract and 70 μ g of embryo nuclear protein extract was loaded per lane (except in lanes 5 and 13 where ~35 μ g protein was loaded). Note that the upper band seen in lanes 3-6 corresponds to a non-specific immunoreaction.

Figure 7 (Panels a-d). Webbed digits of a 2 week-old RAR α null homozygote offspring. Ventral view of left hind-limbs from 2-week old wildtype (a, c) and RAR α homozygote (b,d) animals. Note in b the presence of skin between digits 2, 3, and 4 resulting in a "webbed" limb. Panels c and d correspond to Alizarin red/alcan blue stained skeletons of the limbs shown in panels a and b. Note in d, the absence of any ossified connection between the phalanges indicating that the "webbed" phenotype did not involve any skeletal fusion. Alizarin red/alcan blue staining of skeletons was performed as described elsewhere (Lufkin *et al.*, *Nature* 359:835-841 (1992)).

Figure 8 (Panels a-h). Degenerative lesions in testes of four to five month-old $RAR\alpha$ null homozygotes.

Comparison of histological sections through the testes (panels a-f) and epididymal ducts (panels g-h) of wild type (panels a, d and g) and $RAR\alpha$ null homozygote males (panels b, c, e, f and h), both five month-old. Panel a, the parenchyma of wild type testes ($RAR\alpha$ heterozygote testes were identical) is composed of seminiferous tubules (T) with active spermatogenesis and intertubular spaces containing capillaries (CP) and Leydig cells (L). Note that the aspect of the seminiferous epithelium (or germinal epithelium) varies between tubules at different stages of the spermatogenic cycle; however, all tubules contain primary spermatocytes (C, in panel a). Each of these cells will eventually yield four spermatozooids. Panels b and c, the parenchyma of $RAR\alpha$ homozygote testes shows a patchy pattern of seminiferous tubule lesions. These cover a wide spectrum, ranging from rare tubules with complete spermatogenesis (e.g. T1) to tubules containing only Sertoli cells (e.g. T2) which may be enlarged, thus filling the tubules (e.g., T2 in panel c). A majority of tubules lack primary spermatocytes (C). In addition, the seminiferous epithelium shows numerous large, clear, rounded spaces (vacuole-like, V) and occasional clusters of degenerating spermatogenic cells (large arrow in panel c). In the intertubular spaces, focal hyperplasia of the Leydig cells (L) is observed between atrophic seminiferous tubules (panel c). This hyperplasia is likely to result from the decrease in tubular diameter (compare T3 in panel c with T panel a ; see ref. 40). Panels d, e and f, correspond to high magnification micrographs of the walls of seminiferous tubules. Panel d, in wild type testes, the seminiferous epithelium consists of supporting cells, the Sertoli cells (S) and spermatogenic cells. The spermatogenic cells proliferate from stem spermatogonia (G), located in contact with the basement membrane (B), and differentiate from the periphery towards the lumen of the seminiferous tubules. This process yields different ontogenetically-related cell types arranged in concentric layers, i.e. spermatogonia (G), primary spermatocytes (C), round spermatids (D), and maturing spermatozooids (Z). Panels e and f, are two different aspects of the

seminiferous epithelium in $RAR\alpha$ null homozygote males. Most frequently, the early stages of spermatogenic cell differentiation (e.g. spermatogonia and primary spermatocytes) are missing (panel e: in such a degenerate epithelium spermatogenesis no longer occurs). In rare cases, all stages of spermatogenic cell differentiation, including the round spermatids (D) and maturing spermatozooids (Z) are seen (panel f). Panel g, section through the tail of a wild type epididymal duct; spermatozooids (Z) fill the lumen. Panel h, section through the tail of a $RAR\alpha$ homozygote epididymal duct; the lumen of the duct contains acidophilic (blue) material which is also present within large vacuoles (V) in the epithelium lining the duct (E), possibly as a consequence of extensive cellular absorption; spermatozooids (Z) are occasionally identified in the lumen.

B, basement membrane of the seminiferous tubules; C, primary spermatocytes; CP, capillaries; D, round spermatids; E, epithelium of the epididymal duct; G, spermatogonia; L, Leydig cells; S, Sertoli cells; T, seminiferous tubules; V, vacuoles; Z, spermatozooids. Organs were immersed-fixed in Bouin's fluid. Paraffin sections, 5 μ m thick, were stained with Groat's hematoxylin and Mallory's trichrome.

Figure 9 (Panels a-b). Targeted disruption of the $RAR\beta 2$ locus.

(Panel a) A schematic drawing of the $RAR\beta$ locus is shown at the top, illustrating the alternatively spliced $RAR\beta$ isoforms and their respective promoters (Zelent *et al.*, *EMBO J.* 10:71-81 (1991)). Below is shown the portion of $RAR\beta 2$ genomic locus (Exon 4, E4). At the bottom, the $RAR\beta 2$ targeting vector and the expected structure of the recombinant mutant allele are shown. Note that in the targeting vector the initial 5' XhoI site has been destroyed, and replaced by a Sall site derived from the HSV-TK cassette.

(Panel b) Southern hybridization experiments employing a probe derived from sequences 5' to the targeting vector (probe 1, Fig. 9a) or a full-length neomycin gene probe are shown as indicated. Genomic DNA prepared from targeted ES cells (BH1, BH32 and BH45) and a cell line harboring randomly integrated copies of the targeting vector (BH68) were digested with

- 17 -

KpnI, BamHI and XbaI, and subjected to Southern hybridization. Note on the left that DNAs derived from the targeted cell lines contain in addition to the normal allele (the 6.5kb KpnI fragment, the 20kb BamHI fragment and the 15kb XbaI fragment), the fragments representing the mutant allele (4.3kb for KpnI, 9.5kb for BamHI and 9.0kb for XbaI) which hybridize with probe 1, while the randomly targeted ES cell DNA (BH68) contains only the wild type alleles. B, X, K, Xh, N and S : BamHI, XbaI, KpnI, XhoI, NotI and SalI restriction enzyme sites, respectively.

Figure 10. Southern blot analysis.

Southern blot of DNA derived from one week-old offspring of heterozygous $RAR\beta2$ +/- intercrosses showing the presence of homozygous (-/-, containing just the 4.3kb KpnI fragment), heterozygous (+/-, containing both the 4.3 and 6.5kb KpnI fragments) and wild type (+/-, containing just the 6.5kb KpnI fragment) alleles. Below is shown the distribution of wild type, heterozygous and homozygous mutant one month-old offspring from intercrosses of animals heterozygous for the $RAR\beta2$ mutant allele. Note that the homozygous offspring are present at the expected Mendelian ratio (27%) indicating that there is no post-natal lethality associated with $RAR\beta2$ disruption.

Figure 11. Lack of $RAR\beta2$ protein in $RAR\beta2$ -/- embryos.

Immunodetection experiments employing nuclear extracts prepared from 14.5 dpc wild type (lanes 1 and 6), heterozygous (lanes 2 and 7) and homozygous (lanes 3 and 8) mutant embryos are shown. At left (lanes 1-5), extracts were subjected to direct immunoblotting with a rabbit polyclonal antibody directed against the F-region of $RAR\alpha$. Lanes 4 and 5 contain extracts prepared from Cos-1 cells transfected with $RAR\alpha1$ and $RAR\alpha2$ expression vectors, respectively. Note that the 51kd reactive protein corresponding to the $RAR\alpha1$ isoform is present at similar levels in all three embryo extracts (lanes 1-3). At right, the nuclear extracts were immunoprecipitated with a mouse monoclonal antibody specific for the A2-

region of the RAR β 2 isoform; the protein precipitate was then subjected to immunoblotting with a polyclonal antibody directed against the RAR β F-region (lanes 6-8). A nuclear extract derived from Cos-1 cells transfected with the RAR β 2 expression vector was migrated along side the immunoprecipitates to serve as a size comparison (lane 9). Note the absence of the 51kd RAR β 2 protein in the mutant embryo.

Figure 12 (Panels a-b). RAR β , RAR α and RAR β RNA analysis.

(Panel a) RAR β isoform RNAs in wild type, heterozygote and homozygote mutant embryos. A scheme and the results of RT-PCR experiments are shown. Total RNA prepared from wild type (+/+), heterozygous (+/-) and homozygous (-/-) mutants. 13.5 dpc embryos were analyzed for the presence of RAR β 1/ β 3 and RAR β 2 transcripts. Following RT-PCR amplification, cDNA products were migrated on 2% agarose gels, and subjected to Southern hybridization employing oligonucleotide probes specific for these RAR isoforms. As a control, a cDNA fragment corresponding to the RAR α 1 isoform was amplified (20 cycles) from the 3 RNA preparations, employing a 5'-primer (primer 5, 5'-ATAGCAGTTCCTGCCCCAACAC-3', spanning nucleotides 589-609 in the RAR α 1 A1-region, Leroy *et al.*, 1991 a) and a 3' primer derived from the RAR α C-region (primer 6, 5'-GATGCTTCGTCGGAAGAAGC-3'; spanning nucleotides 908-927) to generate an expected product of 338nt. Note that all 3 embryo RNA preparations contained similar levels of the 338nt RAR α 1 cDNA (shown at the right). To detect the presence of RAR β 2 transcripts (Zelent *et al.*, *EMBO J.* 10:71-81 (1991), a 5'-primer corresponding to nucleotides 504-523 in the RAR β 2 A2-region (primer 2, 5'-GATCCTGGATTTCTACACCG-3') and a 3' primer spanning nucleotides 716-736 in the RAR β C-region (primer 1, 5'-TGGTAGCCCGAGACTTGTCCT-3') were employed to generate a 232nt cDNA product (shown at right, note the product is present in equal amounts in wild type, heterozygous and homozygous RAR β 2 mutant RNAs). To test for the possible presence of wild-type RAR β 2 transcripts in RAR β 2 mutants,

a 5' primer (primer 3, spanning nucleotides 369-388 in the RAR β 2 5'-UT, 5'-GCGAGAGTTTGATGGAGTTC-3') and primer 1 located in the RAR β C-region were employed to amplify a 367nt wild-type product. Note that this product is detectable in wild type embryo RNA, while it is undetectable in homozygote RNAs. To detect the presence of RAR β 1/ β 3 transcripts (Zelent *et al.*, *EMBO J.* 10:71-81 (1991)) in the 3 RNA preparations, a primer located at positions 469-488 in the common RAR β 1/ β 3 A1-region (primer 4, 5'-GAAGCCTGAAGCATGAGCAC-3') and primer 1 located in the RAR β C-region were employed in 30 cycles of amplification to generate products of 306nt for the RAR β 1 isoform and 387 nt for the RAR β 3 isoform (shown at the right). Note that RAR β 1 and β 3 transcripts were present in all 3 RNA preparations.

(Panel b) Determination of RAR α and RAR γ isoform RNA in wild type, heterozygote and homozygote mutant embryos using RNase protection assays. A schematic illustration of the probes and expected protected fragments are shown. Total RNA prepared from 13.5 dpc wild-type (+/+), heterozygous (+/-) and homozygous (-/-) RAR β 2 mutant embryos was employed in the reactions. Riboprobes were as follows (Lufkin *et al.*, *Proc. Natl. Acad. Sci. USA* 90:7225-7229 (1993)): the RAR α 2 antisense probe included the region corresponding to the RAR α 2 initiation codon through the RAR α C-region to generate protected RNA fragments of 379nt for RAR α 2 and 210nt for RAR α 1; the RAR γ 2 antisense probe spanned the RAR γ 2 A2-region through the C-region to generate protected RNA fragments of 345nt (RAR γ 2) and 154nt (RAR γ 1). The histone 4 antisense riboprobe used as an internal control generated a 130nt RNA fragment. Control lanes correspond to the protected RNA fragments obtained with in vitro transcribed sense RNA.

Figure 13 (Panels a-c). RAR β 2 promoter activity in wild type and RAR β 2 nullmutant embryos.

(Panel a) A whole mount of a 10.5 dpc wild-type embryo expressing the RAR β 2/lacZ transgene stained for β -galactosidase activity. Note that

- 20 -

promoter activity is visible in the caudal hindbrain up to the rostral boundary of rhombomere 7 (arrowhead).

(Panels b and c) Whole mounts of $RAR\beta 2$ null embryos (littermates) expressing the $RAR\beta 2$ promoter/lacZ transgene exposed to RA in utero at 10.5 dpc and stained for β -gal activity 4 hours later. Note that the RA-induced rostral shift in promoter activity is similar in the hindbrains of both mutant and wild type embryos (denoted by arrowheads at the approximate level of rhombomere 1 in b and c, see text). Note also the increased labeling of neural crest cells migrating towards the heart (arrow). Abbreviations: ot, otocyst; nt, neural tube.

Figure 14 (Panels a-h). Alteration of the pre-otic hindbrain in RA-treated $RAR\beta 2$ null embryo.

(a-e) show different views of a $RAR\beta 2$ +/+ (wild type) embryo and of a $RAR\beta 2$ -/- mutant embryo which were exposed to RA at 7.25 dpc, collected at 9.0 dpc and hybridized as whole-mounts to a Hoxb-1 probe. (a) and (b), profile views; (c) and (d), hindbrain viewed from the back; (e), ventral view of the mutant embryo. Note that the forebrain vesicles are partially truncated in the wild type embryo (panel a) and almost lacking in the mutant embryo (panel b). The Hoxb-1 signal extends rostrally almost to the extremity of the neuroepithelium. (f-h), three parallel sagittal sections of a $RAR\beta 2$ -/- mutant embryo, which had been subjected to the same RA treatment. The plane of the sections progress from lateral (f) to medial (h) regions. Two neighboring sections hybridized to the Hoxb-1 (middle column) or the Krox-20 (right column) probe are shown under dark-field illumination (signal grains appear white). Only rhombomeres whose boundaries can be tentatively identified are indicated. Hoxb-1 transcripts extend up to the rostral extremity of the neuroepithelium, and a severe truncation of the forebrain is also evident on these sections (panels g and h). ot, otocyst; h, heart.

- 21 -

Figure 15 (Panels a-c). RA-induced limb malformations in wild type and RAR β 2 null mutant fetuses.

Embryos were exposed to RA by maternal gavage with all-trans RA (80mg/kg) at 11.5 dpc, fetuses were collected at 18.5 dpc, and stained with alcian blue and alizarin red to visualize cartilage and bone.

(Panel a) A whole-mount of an alcian blue/alizarin red stained forelimb from a wild type 18.5 dpc fetus. Limbs from RA-treated 18.5 dpc fetuses are shown in b and c.

(Panel b), A forelimb from an RA-treated RAR β 2 null fetus.

(Panel c) A forelimb from an RA-treated wild type fetus. Note that the 5th digit is absent in both wild type and mutant RA-treated fetuses, and that the radii and ulnae are truncated and malformed in similar fashions. H, humerus, R. radius, U, ulna, I, IV and V, 1st, 4th and 5th digit, respectively.

Figure 16 (Panels a-d). Targeting the RXR α Gene.

(Panels a) A map of the RXR α genomic region of interest is shown on top. Probe A is a 2kb BamHI fragment (the 5' BamHI site corresponds to the 5' end of the genomic clone (arrow)); probe B is the 2 kb HindIII-SmaI fragment. Bg: BglII; Sp:SpeI; B:BamHI; H: HindIII; S: SmaI; RV: EcoRV; Xb: XbaI.

(Panel b) Detection of wild-type (WT) and mutant alleles by Southern Blot analysis. Placental DNAs from 12.5 dpc embryos recovered from a cross between heterozygote mice were digested with BamHI and BglII and analyzed by Southern Blot with probe B.

(Panel c) Northern Blot analysis. 30 μ g of total RNA from WT (+/+), heterozygote (+/-) and homozygote (-/-) embryos was analyzed by Northern Blotting with a complete RXR α cDNA probe or the 36B4 constant probe.

(Panel d) Western blot analysis. 15 μ g of nuclear extract prepared from +/+ (lane 2), +/- (lane 3) or -/- (lane 4) 12.5 dpc embryos were analyzed by Western blot with an anti-RXR α polyclonal antibody directed against the N-terminal A/B region. The absence of signal in the -/- lane is not

- 22 -

due to an absence of protein in the corresponding extract, since staining of the same membrane with amido black revealed a similar pattern of proteins in all 3 extracts (not shown).

5 **Figure 17.** Distribution of the weight of wild type and heterozygous animals.

Mice derived from crosses between WT and heterozygous parents were weighed at 2-3 weeks of age. The weight of each animal was expressed as the ratio of its weight relative to the average weight of WT animals of the same litter. Weight ratio were then grouped within classes differing from each other 10 by 0.05 ratio increment (abscissa). The numbers of heterozygote (white bars) and WT animals (black bars) in each class is indicated on the ordinate.

Figure 18 (Panels a-h). External appearance of RXR α null mutant fetuses.

15 Comparison of external features between 14.5 dpc WT (a and c), RXR α -/- (b and d), RXR α /RAR γ (d, e and f) and RXR α /RAR α (c) mutant fetuses.

(Panels a and b) Lateral view of WT (a) and RXR α -/- (b). The arrows in the WT (a) indicate blood vessels which are not visible in the RXR α mutant. The arrow in the RXR α -/- (b) points out the abnormal eye.

20 (Panels c-e) In RXR α -/- (d) and RXR α +/-RAR γ -/- mutants (e) the palpebral fissure, limited by the dorsal and ventral eyelids (DE and VE), is smaller than in the WT (c); and the ventral retina is occulted by the ventral eyelid (VE).

25 (Panel f) In the RXR α -/-RAR γ +/- the palpebral fissure is smaller than in the RXR α -/- (d) and the ventral retina is not visible.

(Panel g) In the RXR α -/-RAR γ -/- fetus there is no palpebral fissure and all the eye is masked by the surface ectoderm and a layer of mesenchyme.

(Panel h) In the RXR α -/-RAR α +/- fetus a unilateral coloboma of the iris (CI) is apparent.

- 23 -

Abbreviations: CI, coloboma of the iris; DE, dorsal eyelid; DR, dorsal retina; P, pupilia; VE, ventral eyelid.

Figure 19 (Panels a-h). Heart defects in $RXR\alpha$ null mutant.

Comparison of the heart in WT (a, c, e and g) and $RXR\alpha^{-/-}$ (b, d, f and h) embryos.

(Panels a and b) Frontal sections of 14.5 dpc fetuses at level of the right atrioventricular canal (AV).

(Panels c and d) Sections of 10.5 dpc embryos at the level of the atrioventricular endocardial cushions (EC).

(Panels e and f) Detail of the ventricular wall in 16.5 dpc fetuses.

Abbreviations: A, atrium; AO, aorta; AV, atrioventricular canal; CL, compact layer; CT, cono truncus; EC, endocardial cushions; VS, ventricular septum; IVC, interventricular communication; LV, left ventricle; PT, pulmonary trunk; RA, right atrium; RV, right ventricle; T, trabeculation of the myocardium wall; TL, trabecular layer; V, ventricle. Magnifications: 55x (a and b), 138x (c, d, e, f, g and h).

Figure 20 (Panels a-d). Electron microscopy of the heart of $RXR\alpha$ null mutants.

Ultrastructural features of the outer portion of the ventricular myocardium in WT (a and b) and $RXR\alpha^{-/-}$ (c and d) 14.5 dpc fetuses.

(Panel a) Low power magnification of the outer compact layer of the WT ventricular myocardium.

(Panel b) Higher magnification of the demarcated area in (a), showing that the myofibrils (M) are present in the cytoplasm, but do not display the sarcomeric organization (S in d). These myocardial cells also contain rough endoplasmic reticulum (RER) and abundant polyribosomes (P). No sarcoplasmic reticulum (SR in d) was visible in this outer layer of the myocardium.

(Panel c) $RXR\alpha^{-/-}$ myocardium comparable area and magnification as shown in (a) for WT.

- 24 -

(Panel d) Higher magnification of the demarcated area in (c). The cells in the outer area of the myocardium exhibit myofibrils; some of them are organized in sarcomeres (S). These cells also display a well developed sarcoplasmic reticulum (SR). Both, sarcomeres and sarcoplasmic reticulum indicate that these cells are at a more advanced stage of differentiation than the compact layer cells in the WT fetus (a and b).

Abbreviations: E, epicardial cell; En, endothelial cell; M, myofibrils; P, polyribosomes; RER, rough endoplasmic reticulum; S, sarcomere; SR, sarcoplasmic reticulum. Magnifications: 4000x (a and c), 25000x (b and d).

Figure 21 (Panels a-f). Eye development in RXR α null mutants.

Comparison of the eye development in WT (a, c and e) and RXR α -/- mutants (b,d and f).

(Panels a and b) Frontal sections through the eye of WT (a) and RXR α -/- (b) 11.5 dpc embryos.

(Panels c and d) Frontal sections of 12.5 dpc WT (c) and RXR α -/- (d) embryos.

(Panels e and f) Frontal sections of 15.5 dpc fetuses.

Abbreviations: AC, anterior chamber; C, cornea; C*, thicker cornea; CON, coloboma of the optic nerve; DE, dorsal eyelid; DJ, dorsal conjunctival sac; DR, dorsal retina; F, persistent retrolenticular membrane; FE, fused eyelids; J, conjunctival sac; L, lens; ON, optic nerve; RE, root of the eyelid; SE, surface ectoderm; V, vitreous body; VE, ventral eyelid; VJ, ventral conjunctival sac; VR, ventral retina. Magnifications: 55x (e and f), 138x (a, b, c and d).

Figure 22 (Panels a-l). Eye defects in RXR α -/-, RXR α /RAR γ and RXR α /RAR α double mutants.

Frontal sections through the eye of 12.5 dpc (e), 14.5 dpc (a, b, c, d, f, g, h, i and j) and 16.5 dpc (k and l) fetuses. Genotypes as indicated.

Abbreviations: AC, anterior chamber; C, cornea; C*, thicker cornea; CE, corneal epithelium; CL, corneal-lenticular stalk; CON, coloboma of the

- 25 -

optic nerve; CS, corneal stroma; EOM, extraocular mesenchyme; DE, dorsal eyelid; DJ, dorsal conjunctival sac, DR, dorsal retina; ER, eversion of the retina; F, persistent retrolenticular membrane; L, lens; ON, optic nerve; PR, pigment retina; RE, root of the eyelid; SC, sclera; SE, surface ectoderm; V, vitreous body; VE, ventral eyelid; VJ, ventral conjunctival sac; VR, ventral retina. Magnifications: 138x (a, b, c, d, e, f and g), 280x (h, k and l), 550 x (i and j).

Figure 23 (Panels a-i). Three dimensional computer reconstruction of WT, $RXR\alpha^{-/-}$ and $RXR\alpha^{-/-}RAR\gamma^{-/-}$ eyes.

Three dimensional computer reconstruction from serial frontal histological sections of the eye of WT (a, d and g), $RXR\alpha^{-/-}$ (b, e and h) and $RXR\alpha^{-/-}RAR\gamma^{-/-}$ (c, f and i) 14.5 dpc fetuses.

(Panels a, b and c) Dorsal views of the eye; the surface ectoderm has been removed.

(Panels d, e and f) External views of the eye in which the surface ectoderm has been rendered transparent.

(Panels g, h and i) Ventral views of the eye without the surface ectoderm. In the $RXR\alpha^{-/-}$ (b, e and h) it is possible to appreciate the rotation of the eye around the dorso-ventral axis toward the snout. Note in the external view that the dorsal and ventral eyelids (DE and VE) are closer than in the WT eye (d). In the external view of the $RXR\alpha^{-/-}RAR\gamma^{-/-}$ eye (f) it is possible to see the surface ectoderm invagination of the cornea lenticular stalk (CL) and the eversion of the retina (ER).

Abbreviations: CL, cornealenticular stalk; DE, dorsal eyelid; ER, eversion of the retina; L, lens; PR, pigment retina; VE, ventral eyelid.

Magnification: 49x (a-i).

Description Of The Preferred Embodiments

The present invention provides; 1) mice which are deficient in expressing either functional, normal levels, or detectable levels of one or more members of the RAR or RXR family of receptors, 2) mice heterozygous for such a deficiency, 3) cell lines, preferably pluripotent or totipotent cell lines, which are heterozygous or homozygous for such deficiency, and 4) methods of using the cell lines and mice of the present invention, or tissues derived therefrom, to identify antagonists and agonists of RXR or RAR receptors.

The present invention is based on the novel observation that homologous recombination can be used to replace one or more of the sequences encoding a member of the RAR or RXR family of receptors with a sequence which either prevents expression of all of the isoforms of a given subtype or a specific isoform of RAR or RXR receptor, or with a sequence which encodes an altered form of the receptor. The examples provided below demonstrate that gene replacement techniques can be used to knock out or alter the expression of one or more of the RAR or RXR family of receptors.

In one embodiment, the present invention provides mice and cell lines which have been altered to contain a sequence which confers a deficiency in the normal expression of at least one member of the RAR or RXR class of proteins. The cell lines and mice of the present invention can be heterozygous or homozygous for the desired trait, provided that the mice or cell lines contain the altered RAR or RXR coding sequence.

As used herein, a mouse or cell line is said to be altered to contain a sequence which conveys a deficiency in the normal expression of a member of the RAR or RXR class of receptors if recombinant techniques are utilized to insert, delete or replace sequences encoding for, or directing the expression of, one or more members of the RAR or RXR family of receptors. The insertion, deletion or replacement within such sequences has the effect of altering the normal level of expression of the given sequence or altering the activity of the protein which is expressed.

Mice can be altered such that the mouse expresses a lower level of the protein when compared to a non-altered mouse (in some cases a mouse "deficient" in expressing normal levels of a protein will be incapable of expressing detectable levels of the given protein). In some instances, where a mouse is altered such that a target gene is deleted or a large insertion is generated within the target sequence, the mouse will not produce detectable levels of the given receptor. However, in some instances it may be possible for extremely low quantities of the given receptor to be produced, although such product may, in itself, be inoperative, or not functional in its usual physiological actions.

As used herein, "normal expression" is defined as the level of expression which is present in a wild-type or non-altered animal. A variety of techniques known in the art can be used to quantitate the level at which a given protein is expressed. These include, but are not limited to immunological techniques such as an ELISA, RIA, or western blot, or quantitative analytical techniques such as spectroscopy or flame chromatography.

Alternatively the mice of the present invention can be altered so as to express an altered form of the given protein. Mice can be altered such that a specific mutation is introduced into a given region of a subtype or specific isoform of a RAR or RXR. Alternatively, mice can be altered such that the subtype or specific isoform of a RAR or RXR is altered (for example, the sequence encoding RAR- γ can be replaced with sequence encoding RAR- α) and the subsequent effects observed.

As used herein, the RAR and RXR class or family of receptors is defined as proteins which share the overall structure and sequence organization of members of the nuclear receptor proteins thus far identified as RAR or RXR receptors. These include, but are not limited to, all the various subtypes and isoforms of RAR and RXR, for example the alpha, beta, and gamma subtypes, and the RAR- α 1, RAR- α 2, RAR- β 1, RAR- β 2 and RAR- γ 1 and RAR- γ 2 isoforms disclosed in Krust *et al.*, *Proc. Natl. Acad. Sci. USA* 86:5310-5314 (1989); Giguère *et al.*, *Mol. Cell. Biol.* 10:2335-2340 (1990),

Kastner *et al.*, *Proc. Natl. Acad. Sci. USA* 87:2700-2704 (1990), Leroy *et al.*, *Embo J.* 10:59-69 (1991), Zelent *et al.*, *Embo J.* 10:71-81 (1991) herein incorporated by reference.

5 As used herein, a "subtype" of RAR or RXR receptor is identified by the presence of a subtype specific sequence which occurs within the A, B and/or D regions of the receptor. All isoforms from a given organism of a specific RAR or RXR subtype, for example all isoforms of human RXR- β , possess a conserved sequence within one of these regions which defines the subtype.

10 As used herein, an "isoform" of a particular subtype of RAR or RXR receptor is identified by sequence heterogeneity which is present in the A region of the RAR or RXR receptor. The various isoforms of a RAR or RXR receptor from a given organism will possess differing A region sequences.

15 Using the procedures outlined in the Examples presented below as well as those known in the art, one of ordinary skill can generate vectors for altering the expression of one or more subtypes or specific isoforms of RAR or RXR receptors without undue experimentation. Specifically, an individual wishing to use homologous recombination (HR) to disrupt a specific subtype or isoform of RAR or RXR first uses the sequences encoding one of the
20 various RARs or RXRs disclosed in Krust *et al.*, *Proc. Natl. Acad. Sci. USA* 86:5310-5314 (1989), Giguère *et al.*, *Mol. Cell. Biol.* 10:2335-2340 (1990), Kastner *et al.*, *Proc. Natl. Acad. Sci. USA* 87:2700-2704 (1990), Leroy *et al.*, *Embo J.* 10:59-69 (1991), Zelent *et al.*, *Embo J.* 10:71-81 (1991) to isolate a genomic fragment harboring a region of the receptor of interest, modifies a
25 certain region of the genomic fragment to create a null allele for the receptor, and then uses this modified genomic sequence to perform HR. For example, Example 1 describes vectors which either selectively inactivate the entire RAR- γ subtype (these vectors contain modified sequences from the B region of RAR- γ) or inactivate only the RAR- γ 2 isoform (these vectors contain
30 modified sequences from the A region of RAR- γ 2). Example 2 describes vectors which either selectively inactivate the entire RAR- α subtype (these vectors contain modified sequences from the B region of RAR- α) or inactivate

only the RAR- α 1 isoform (these vectors contain modified sequences from the A region of RAR- γ 1).

The mice and cell lines of the present invention are preferably obtained by methods known in the art as homologous recombination (HR). This method has long been known in lower eukaryotes (e.g. yeast), and has recently been described for the mouse (for review, see Capecchi, M., *TIG* 5(3):70-76 (1989) and also see Smithies, O., *et al.*, *Nature* 317:230 (1985); Zijlstra, M., *et al.*, *Nature* 342:435 (1989); Schwartzberg, P.L., *et al.*, *Science* 246:799 (1989); DeChiara, T.M., *et al.*, *Nature* 345:78 (1990)).

Homologous recombination essentially comprises isolating genomic sequences containing the target gene, employing known genetic engineering techniques to mutate or otherwise disable or modify the gene, and then reintroducing the gene into the relevant species. This is achieved by preparing a culture of pluripotent, or totipotent, cells, typically taken from embryos (ES cells). The advantage of these cells is that they can be successfully cultured for a large number of generations under conditions in which they will not differentiate and can be reintroduced into recipient embryos.

A sample of mouse pluripotent embryonic cells (ES-D₃), which can be used to reproducibly obtain the mice and cell lines of the disclosed invention, was deposited under the Budapest Treaty at the ATCC on May 6, 1994. The ATCC designation for this cell line is CRL11632.

Typically the technique of electroporation, is used to render the ES cells capable of taking up exogenous DNA. The modified gene is then introduced, in a suitable manner, to these cells. Once taken up, recombination may occur, although this may be by random integration as well as by homologous recombination.

To select cells in which a recombination event has taken place, a selectable marker sequence may be used. For example, it is well known to employ the bacterial Neo gene to confer resistance to neomycin, or an analogue thereof, such as G418. The marker gene may be inserted in the gene

- 30 -

to be modified, thereby disabling the target gene, while providing a positive selectable marker. Clones which are Neo⁺ have integrated the vector.

To further select homologous recombinants, the ends of the modified gene may have other markers inserted, such as the Herpes Simplex Virus thymidine kinase (HSVTK) gene. In a HR event, the HSVTK genes will not be recombined, and the marker will not be transferred. Therefore, the desired recombinant will be resistant to, for example, Gancyclovir, which is converted into a toxic metabolite when the HSVTK gene product is present (after a non-homologous recombination event).

Correct clones may be identified by the technique of PCR or by genomic Southern blotting.

Subsequently, when a suitable clone has been identified, the ES cells may be injected into early-stage embryos, (blastocysts), and reintroduced into a pseudopregnant female.

Chimeric animals will generally result from at least some of these embryos, their tissues deriving in part from the selected clone. Thus, the germ-line may also be chimeric, spermatozoa or ova containing the modified gene. Progeny deriving from such germ cells will be heterozygous for the gene.

The heterozygous progeny can be cross-bred to yield homozygous animals. Confirmation of the allelic structure of the mice can be ascertained by Southern blotting, for example.

The present invention also envisages cell lines suitable for generating mice of the invention, and techniques for generating such lines and mice.

Thus, to obtain mice according to the present invention, one skilled in the art can use the strategy of homologous recombination (HR) in embryonic stem cells (ES cells) to replace the wild-type sequences encoding a member of the RAR or RXR family of receptors with an altered sequence.

The absence of one or more members of the RAR or RXR class of proteins in a cell line or animal allows one skilled in the art to screen for genes and agents which can restore the altered mice to a wild-type phenotype,

- 31 -

as well as to screen for agents which act as agonists or antagonists of one or more members of the RAR or RXR class of receptors.

The mice and cell lines of the present invention allows the investigation, at the cellular level as well as at the *in vivo* level of a system which lacks one or more subtypes or specific isoforms of RAR or RXR receptors. This will allow researchers to establish the importance of each of the various subtypes or isoforms of RAR or RXR receptors.

The mice and cell lines of the present invention may also be deficient in the expression of other genes, and thus provide the opportunity to study the interactions of RAR and RXR proteins with other proteins or the effects these proteins have on RA dependent gene expression.

Thus, it will be appreciated that there are many uses to which the mice and cell lines of the present invention may be put. They are particularly useful in studying any aspect of RA mediated gene expression and tissue specific expression of various RAR/RXR receptors. Also, as described below, the mice and cell lines of the present invention may be used to identify agonists and antagonists of specific members of the RAR/RXR class of receptors.

The mice and cell lines of the present invention can be used to assay for agents which act as antagonists or agonists of specific members of the RAR or RXR class of receptors. In general, the agent which is to be tested will be incubated with one or more of the cell lines or mice of the present invention, or tissues derived therefrom, and the level of binding of the agent is determined or the effect the agent has on development or gene expression is monitored.

As used herein, incubate is defined as contacting the compound or agent under investigation with the appropriate cell or tissue of the invention; or administering the agent or compound to the appropriate mouse of the invention via any one of the well-known routes of administration including enteral, intravenous, subcutaneous, and intramuscular.

For example, the cell lines and mice of the present invention, or tissues derived therefrom, can be used in an assay system comprising the steps of:

- 32 -

- (a) incubating an agent with one or more of the cell lines or mice of the present invention, or cells or tissues derived therefrom; and
- (b) determining whether the agent binds to the cells, tissues, or mice, or determining the effects the agent has on development or gene expression.

5 In performing such an assay, one skilled in the art will be able to determine which subtype or isoform of RAR or RXR receptor an agent binds to, and hence determine what specific receptor(s) are utilized by a given compound. Additionally one can determine in which tissues a given RAR/RXR receptor is active.

10 The agents screened in the above assay can be, but are not limited to, peptides, carbohydrates, and vitamin derivatives. The agent can be selected, and screened at random, rationally selected or rationally designed using protein modeling techniques.

15 For random screening, agents such as peptides, carbohydrates, or derivatives of RA, are selected at random and are assayed for their ability to bind to one of the heterodimers present in mice or cell lines described in the present invention using either direct or indirect methods.

20 Alternatively, agents may be rationally selected. As used herein, an agent is said to be "rationally selected" when the agent is chosen based on the physical structure of a known ligand of the heterodimer. For example, assaying compounds possessing a retinol like structure would be considered a rational selection since retinol like compounds will bind to a variety of the heterodimers.

25 Since highly purified RAR and RXR proteins are now available, X-ray crystallography and NMR-imaging techniques can be used to identify the structure of the ligand binding site present on these proteins. Utilizing such information, computer modeling systems are now available that allows one to "rationally design" an agent capable of binding to a defined structure (Hodgson, *Biotechnology* 8:1245-1247 (1990)), Hodgson, *Biotechnology* 30 9:609-613 (1991)).

- 33 -

As used herein, an agent is said to be "rationally designed" if it is selected based on a computer model of the ligand binding site of the RAR or RXR protein.

5 In one aspect of the above-described assay, the cell line or mouse, in addition to being altered in the expression of one or more of the members of the RAR or RXR class of receptors, is altered such that it contains a marker sequence, such as luciferase, beta galactosidase, or chloramphenicol acyltransferase, operably linked to a RAR or RXR response element (RARE or RXRE). The agent which is to be tested is incubated with the altered cell
10 or mouse, or tissues derived therefrom, and the expression of the reporter sequence is assayed. In this fashion, agents can be identified which are capable of either stimulating or inhibiting the expression of a DNA sequence which is controlled by a specific RARE or RXRE.

15 The following Examples serve only to illustrate the invention, and are not to be construed as in any way limiting on the invention.

Example 1

Function of Retinoic Acid Receptor γ (RAR γ) in the Mouse

Experimental Procedures

RAR γ 2 and RAR γ Homologous Recombination Targeting Vectors

20 Both RAR γ 2 and RAR γ were targeted using the "replacement" strategy (Capecchi, *Science* 244:1288-1292 (1989)). For the RAR γ 2 disruption, the construct was prepared as follows; a 9 kb EcoRI fragment containing exon 5 (RAR γ 2 specific A domain) was subcloned from phage λ G1 mRAR γ (Kastner
25 *et al.*, *Proc. Natl. Acad. Sci. USA* 87:2700-2704 (1990)) into the vector pTZ19U (Pharmacia). Most of the RAR γ 2 5' untranslated region and part of the sequences encoding the A2 region (nucleotides 81 to 342; numbering as

- 34 -

in Kastner *et al.*, *Proc. Natl. Acad. Sci. USA* 87:2700-2704 (1990)) were then deleted by site-directed mutagenesis. The GTI-II NEO cassette was obtained as a NotI fragment by PCR amplification of the plasmid p566 (Lufkin *et al.*, *Cell* 66:1105-1119 (1991)) using the oligonucleotides 5' ATGCGGCCGCGGTACCCAGATCTCGGG (SEQ. ID NO. 1) and 5' ATGCGGCCGCTCATTTTCGAACCCCAGAG (SEQ ID NO. 2) and was subcloned into the EagI site present in exon 5. Thus, in this construct, part of the RAR γ 2 5' untranslated region and A2 region sequences up to amino acid 19 (which includes all methionines in this region) were replaced with the GTI-II NEO sequences. The genomic fragment, containing the NEO insert, was subsequently subcloned into a HSV TK expression cassette [derived from p511 (Lufkin *et al.*, *Cell* 66:1105-1119 (1991))]. This construct, designated γ 9.1, had 7.5 kb and 1.5 kb of homologous sequence 5' and 3' of the NEO insertion respectively. The construct was linearized at the unique Sall or SfiI sites present in the polycloning site prior to electroporation of D3 ES cells. For targeting all isoforms of RAR γ , a 6 kb EcoRI subclone, contiguous with the fragment used to disrupt RAR γ 2, was subcloned from λ G1 mRAR γ into pTZ19U in which the KpnI site in the polylinker had been destroyed by blunting with T4 DNA polymerase. A NEO cassette derived from p566 (Lufkin *et al.*, *Cell* 66:1105-1119 (1991)) was cloned into the unique KpnI site present in E8 which contains the B region common to all RAR γ isoforms (amino acid 75 of RAR γ 1). A HSV TK expression cassette, derived from p511 (Lufkin *et al.*, *Cell* 66:1105-1119 (1991)), was then cloned into the unique Sall site present in the polycloning site of pTZ19U, to yield the final targeting vector designated γ 6.1. This construct had 2.8 kb and 3.2 kb of homologous sequence 5' and 3' of the NEO insert respectively, and was linearized at the unique HindIII site in the polylinker prior to electroporation of D3 ES cells.

Electroporation conditions, cell culture, G418 and gancyclovir selection, DNA preparation, PCR analysis, Southern blot analysis and generation of chimeric mice were as described previously (Lufkin *et al.*, *Cell* 66:1105-1119 (1991)). Initial screening for the RAR γ 2 disruption was

- 35 -

performed by the PCR using DNA pooled from 5 separate ES cell clones, with the nested primers 5' ATACCCAGATATTTCTGACTCAG 3' (SEQ. ID NO. 3); 5' CAGATGCCAGCCAATGTGCCCA 3' (SEQ. ID. NO. 4) (genomic sequence 3' of boundary of homologous recombination) and 5' ACCGCTTCCTCGTGCTTTACGGTA 3' (SEQ. ID NO. 5); 5'GCCGCTCCCGATTTCGCAGCGCAT3' (SEQ. ID NO. 6) (NEO sequence). DNA from individual colonies from pools positive by the PCR were then analyzed by genomic Southern blotting. Probe 1 corresponds to a 1.6 kb BamHI-EcoRI genomic fragment derived from phage λ G1 mRAR γ respectively; probe 2 corresponds to exon seven of RAR γ 6 (Kastner *et al.*, *Proc. Natl. Acad. Sci. USA* 87:2700-2704 (1990)). Additional probes (not shown) including NEO and HSV tk were used to establish the fidelity of the recombination events.

RNase protection analysis

Total RNA was prepared from either day 10.5 or 13.5 p.c. embryos or various tissues by the single-step guanidinium-isothiocyanate-phenol technique (Chomczynski and Sacchi, *Analytical Biochem.* 162:156-159 (1987)) with the addition of a second phenol-chloroform extraction step. Approximately 40 μ g of RNA was used per hybridization. The conditions for the preparation of probes and the hybridization reaction were essentially as described previously (Sambrook *et al.*, in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1987).

Histological and skeletal analysis

For whole mount skeletal analysis, fetuses were collected at day 18.5 p.c. and stored at -20°C. Skeletons were prepared as previously described (Lufkin *et al.*, *Nature* 359:835-841 (1992)). For histological analysis, fetuses were skinned and then fixed in Bouin's fluid. Paraffin sections, 7 μ m thick,

were stained with Groat's hematoxylin and Mallory's trichrome. Organs were fixed in 10 % formalin, and histological sections, 5 μ m thick, were stained with hematoxylin and eosin.

Retinoic acid treatment.

5 RAR $\gamma^{+/-}$ heterozygote animals were mated overnight and the presence of a vaginal plug at noon the next day considered as day 0.5 p.c. At various times, pregnant females received a single dose of RA dissolved in sunflower seed oil at a final delivery of 100 mg RA per kg animal.

Results

10 ***Homologous recombination and germ line transmission***

 An approach based on positive-negative selection (Capecchi, *Science* 244:1288-1292 (1989)) was employed to create a null allele in embryonic stem (ES) cells for either RAR γ 2 or all RAR γ isoforms (Kastner *et al.*, *Proc. Natl. Acad. Sci. USA* 87:2700-2704 (1990); Fig. 1a). For disruption of RAR γ 2, a
15 neomycin phosphotransferase expression cassette (NEO) was subcloned into the RAR γ 2-specific exon 5 at the position corresponding to amino acid 19 (Kastner *et al.*, *Proc. Natl. Acad. Sci. USA* 87:2700-2704 (1990)). This
20 targeting vector yielded a total of six "disrupted" ES cell clones (from a total of 186), of which three were injected into blastocysts to derive chimeras. Two
 chimeras (derived from EA36 and EB1 ES clones) tested positive for germ line transmission (data not shown); although similar results were obtained for
 offspring from either line, only those corresponding to EB1 are presented here. Southern blot and RNase protection analysis indicated that homologous
25 recombination resulted in the planned disruption; secondary integration events, duplications or rearrangements were not detected (Fig. 1b and d, and data not
 shown). Due to the nature of this disruption, some truncated RAR γ protein could possibly be synthesized via reinitiation of translation at the AUG codon

- 37 -

located at position 41 in the B region of the mutated receptor transcript. It is important to note that this putative protein would also lack the distinctive A2 region of the RAR γ 2 isoform, and therefore, that its synthesis should still allow an analysis of any specific function of this isoform.

5 The whole RAR γ gene was disrupted using a construct in which the NEO cassette was inserted in exon 8 (B region), which is common to all RAR γ isoforms (at amino acid 75 of RAR γ 1; Fig. 1a and Kastner *et al.*, *Proc. Natl. Acad. Sci. USA* 87:2700-2704 (1990)). Eleven "disrupted" ES cell clones were obtained using this construct (from a total of 72 colonies), of
10 which nine were injected into blastocysts to derive chimeras. One chimera (AA71) passed the mutation to its offspring, and all results presented were derived from this line. Southern blot and RNase protection analysis showed that, as for RAR γ 2, the planned disruption was successful (Fig. 1b and d, and data not shown). Reinitiation of translation of the mutated RAR γ transcript
15 could theoretically occur via the AUG corresponding to amino acid position 122 of RAR γ 1. However, since the corresponding methionine is located between the two zinc fingers of the C region, the putative resulting protein would be non-functional, as it would contain only the second zinc finger, and therefore would not be able to bind to RA response elements (Leid *et al.*, *Cell*
20 68:377-395 (1992b)).

RAR γ 2 null homozygotes are apparently normal

Analysis of RAR γ 2^{+/-} x RAR γ 2^{+/-} or RAR γ 2^{-/-} x RAR γ 2^{+/-} offspring gave a Mendelian distribution for the mutation (Table I). Both RAR γ 2^{-/-} males and females were fertile (Table I, RAR γ 2^{-/-} x RAR γ 2^{+/-} crosses, and data not
25 shown), and appeared indistinguishable from their wild-type (WT) or RAR γ 2^{+/-} littermates. Histological examination of serial sections and whole mount skeletal analysis of day 18.5 p.c. RAR γ 2 null fetuses delivered by caesarian did not reveal any abnormalities.

The lack of an overt phenotype for the RAR γ 2 disruption could not be attributed to alternative splicing of the mutated transcript, as RNase protection analysis of RNA extracted from either RAR γ 2^{-/-} day 10.5 p.c. whole embryos or adult lung showed only the predicted transcript, nor were transcripts encoding any other RARs apparently induced to compensate for the loss of this receptor (Fig. 1c and d, and data not shown).

Table 1

Offspring Viability of RAR γ Mutant Mice

Genotype of RAR γ ^{+/+} X RAR γ ^{+/+} Crosses			
	+/+	+/-	-/-
Day 18.5 p.c.	69 (1.0)	146 (2.1)	65 (0.9)
1-3 weeks old	142 (1.0)	297 (2.1)	73 (0.5)
1 month old	140 (1.0)	295 (2.1)	53 (0.4)
3 months old	138 (1.0)	292 (2.1)	28 (0.2)
Genotype of RAR γ 2 ^{+/+} X RAR γ 2 ^{+/+} Crosses			
	+/+	+/-	-/-
1-2 months old	41 (1.0)	93 (2.3)	38 (0.9)
Genotype of RAR γ 2 ^{+/+} X RAR γ 2 ^{+/+} Crosses			
	+/+	-/-	
1-2 months old	47 (1.0)	48 (1.0)	

Table I. Offspring viability of RAR γ mutant mice. Genotype analysis was performed using DNA prepared from either the placenta (day 18.5 p.c.) or tail tips (1 week- 3 months p.p.). The number of RAR γ or RAR γ 2 WT offspring or fetuses was arbitrarily assigned a value of 1.0, and the relative ratios of heterozygote and homozygote animals calculated accordingly (number in parenthesis).

Growth-deficiency and early lethality of RAR γ null homozygotes

Disruption of all isoforms reduced by about 50% the expected number of RAR $\gamma^{-/-}$ offspring when genotyping was performed at 1-3 weeks of age (Table I). Moreover, these RAR $\gamma^{-/-}$ animals had a higher mortality rate than their RAR $\gamma^{+/+}$ or WT littermates, with less than 40% surviving to 3 months of age (Table I). Interestingly, the mutation was not embryonic lethal, as a Mendelian distribution was observed for day 18.5 p.c. fetuses delivered by caesarian section. Moreover, the RAR γ null fetuses did not exhibit any visible abnormalities, and survived as well as their littermates for more than 24 hours when segregated from dams. However, approximately 50 % of these RAR γ null fetuses were selectively cannibalized within 24 hours when placed with adoptive mothers.

4-5 day post-partum (p.p.) RAR $\gamma^{-/-}$ animals were often growth-deficient, typically 40-80% the weight of heterozygote or WT siblings (data not shown), yet displayed no obvious malformations (with the exceptions noted below) at any stage examined to date. However, these smaller RAR γ null animals exhibited a higher mortality rate in the following 1-2 months (Table I), which resulted in the remaining RAR $\gamma^{-/-}$ animals having a body weight which approached that of WT littermates at 3 months (data not shown).

The expression of RAR α isoforms was not grossly affected by RAR γ disruption, as seen by RNase protection analysis using RNA extracted from day 13.5 p.c. embryos or adult lung, testes, vas deferens, seminal vesicles and prostate (Fig.1d, and data not shown). Neither did *in situ* hybridization analysis reveal any obvious changes in the expression pattern of RAR α or β transcripts in day 10.5 p.c. RAR $\gamma^{-/-}$ embryos. Furthermore, when compared with WT or RAR $\gamma^{+/+}$ embryos, the expression pattern of a RA-inducible RAR β 2 P2 promoter LacZ reporter (Mendelsohn *et al.*, *Development* 113:723-734 (1991)) was not altered at day 9.0 or 13.5 p.c. in RAR $\gamma^{-/-}$ embryos from either normal or RA-treated dams (data not shown).

Homeotic transformations in RAR γ null animals

25 out of 29 day 18.5 p.c. RAR γ ^{-/-} fetuses exhibited various malformations of the axial skeleton at different frequencies, none of which were found in wild-type littermates (Table II). Homeotic transformations included: (i) anterior transformation of the axis (second cervical vertebra, C2) to an atlas (first cervical vertebra, C1) identity as evidenced by a thickening of the neural arches and the presence of an ectopic anterior arch of the atlas (AAA*) fused to C2 ("C1" in Fig. 2, compare panel a with b and d; however, note that the axis dens was still present and therefore that the C2 to C1 transformation was not complete); (ii) unilateral anterior transformation of C7 to a sixth vertebral identity ("C6") with a concomitant unilateral anterior transformation of C6 to a fifth cervical identity ("C5"), as evidenced by the presence of a tuberculum anterior ("TA") and a foramina transversarium ("FT") on C7 (WT C7, unlike C3 to C6 vertebrae, has no *foramina transversaria*, see panel i), and lack of a tuberculum anterior on C6, respectively (compare Fig. 2a with 2b; and 2i with 2j, k and l; note in panel 2j that the C7 to C6 transformation is almost bilateral, thick arrow); and (iii) anterior transformation of the eighth thoracic vertebra to a seventh thoracic identity, resulting in eight instead of seven vertebrosteral ribs (compare Fig. 2e with 2f and g). This last transformation was also found at a low frequency in heterozygote animals (Table II), indicating that the specification of this segment could be particularly sensitive to RAR γ gene dosage effects.

Table II

<i>Skeletal Abnormalities in RARγ^{-/-} Offspring</i>			
	RAR γ Genotype		
	+/+	+/-	-/-
Fetuses Examined at Day 18.5 p.c.	33	36	29
Abnormal Fetuses	0	3 (*%)	25 (86%)
5 Vertebral Abnormalities			
Transformations			
Basioccipital/C1 AAA fusion ^(a)	0	0	8 (25%)
C2 to C1 ^(c)	0	0	5 (17%)
C7 to C6 ^(b)	0	0	4 (13%)
10 8 vertebrosteral ribs ^(d)	0	3 (11%)	10 (33%)
Ribs 1 & 2 fused ^(d)	0	0	4 (13%)
Malformations			
C1 bifidus ^(b)	0	0	2 (7%)
C2 bifidus ^(b)	0	0	3 (10%)
15 C1 & C2 fused ^(c)	0	0	1 (3%)
C2 & C3 fused ^(b)	0	0	4 (13%)
Malformed Tracheal Cartilage	0	0	29 (100%)

Table II. Skeletal abnormalities in RAR γ null mutant offspring. Genotyping was performed using DNA from the placenta of day 18.5 p.c. offspring. Skeletons were prepared, and abnormalities scored under a dissecting microscope. Malformations of symmetrical structures were scored whether they were bilateral or unilateral. (a) includes 3 fetuses analyzed by histological sections (b) only or mostly unilateral (c) bilateral (d) unilateral or bilateral.

Two malformations present in RAR γ ^{-/-} animals which could also represent homeotic transformations included fusion of the first and second ribs (Fig. 2c and d), and fusion of the basioccipital bone to the anterior arch of the atlas (compare Fig. 2a and b, Figs. 3a-d). The fusion of the first and second

ribs may represent an anterior transformation of the second thoracic vertebra to a first thoracic identity. However, the characteristic processus spinous was still present on the second thoracic vertebra. The fusion between the basioccipital bone and the anterior arch of the atlas may result from the abnormal persistence of the first hypochordal bar. Hypochordal, or subnotochordal, bars are normally formed by the ventral union of the left and right cephalic prevertebral components (derived from the sclerotomic mesenchyme) beneath the notochord (Hamilton, *In an Introduction to Embryology*, B.H. Willier, ed. (New York: Henry Holt) 1952). In mouse embryos, three hypochordal bars are formed which are localized opposite the ventral side of the first and second cervical prevertebrae and are visible in day 12 p.c. embryos as mesenchymal condensations distinct from those of the bodies of these prevertebrae (Theiler, *In Advances in Anatomy, Embryology and Cell Biology*, Volume 112, F. Beck, W. Driz and J.E. Pauly, eds. (Heidelberg: Springer Verlag), pp. 1-99 (1988)). The first (rostral most) and third hypochordal bars become incorporated, while still mesenchymal, into the caudal edge of the basioccipital bone and into the body of the axis, respectively. The second hypochordal bar normally persists, chondrifies and subsequently ossifies to form the anterior arch of the atlas. In $RAR\gamma^{-/-}$ day 18.5 p.c. fetuses the first hypochordal bar often persists as a small median cartilage (H1 in Fig. 3, panels b and d) fused to the caudal edge of the basioccipital bone (BO). In this situation the caudal edge of the basioccipital bone becomes attached to the lateral mass of atlas (C1L) by the transverse ligament of the atlas (TL, see panel b), and/or is fused through an osseous bridge to the anterior arch of the atlas (panel d; see also Fig. 2 panel b). This persistence can be interpreted as a posterior homeotic transformation of the basioccipital bone (i.e., this bone has acquired an additional structure which is characteristic of the wild-type atlas), whereas the persistence of the third hypochordal bar, which is frequently observed in $RAR\gamma^{-/-}$ fetuses (see Fig. 2 panels b and d, AAA*), may correspond to an anterior homeotic transformation (i.e., the third hypochordal bar acquires the characteristics of the second hypochordal bar). Additional homeotic transformations of the axial

skeleton may be present in $RAR\gamma^{-/-}$ animals that cannot be identified due to lack of morphological landmarks, as has been previously discussed (Kessel and Gruss, *Cell* 67:89-104 (1991)), e.g. it is possible that the entire cervical region of the homozygote null mutant displayed in Fig. 2b has undergone an anterior homeotic transformation.

Malformations of cartilage-derived structures in $RAR\gamma$ null mice

Additional malformations involving cartilage-derived structures in $RAR\gamma^{-/-}$ animals included (i) ossified fusions between the neural arches of either C1 and C2 or C2 and C3 (compare Fig. 2a with 2b and h, thick arrows, and data not shown); (ii) bifidus of the neural arch of the first or second cervical vertebrae (Table II, and data not shown); and (iii) malformation of the tracheal cartilaginous rings (fusion of the cartilaginous rings on the ventral side of the trachea and disruption of the rings; compare T in Fig. 2c with 2d and f, and 3e with 3f). Note that this latter malformation was found in all of the $RAR\gamma^{-/-}$ animals (Table II). No other bone or cartilage malformations were noted.

Glandular and epithelial defects in $RAR\gamma$ null mice

$RAR\gamma^{-/-}$ animals were often found with closed and encrusted eyelids on one or both sides (data not shown). This phenotype became apparent approximately 2 weeks after birth (ie. after normal eye opening; data not shown). Subsequent analysis of these animals revealed in some cases a bilateral absence of the Harderian gland epithelium (in 2 of 8 $RAR\gamma^{-/-}$ offspring examined; the ocular Harderian glands, together with the palpebral glands, provide lubrication of the edges of the eyelids). In other cases, one of the Harderian glands was normal, whereas the epithelium of the contralateral gland was partially or totally absent (in 2 of 8 $RAR\gamma^{-/-}$ offspring examined, Fig. 4, panels m to o, and data not shown). This malformation is probably congenital, as histological analysis of a day 18.5 p.c. $RAR\gamma^{-/-}$ fetus

revealed bilateral absence of the Harderian glandular epithelium (data not shown). In other animals exhibiting the "closed and encrusted eyelid" phenotype the Harderian glands appeared histologically normal, thus suggesting that they could be simply functionally impaired. Wild type and heterozygote animals never exhibited this phenotype.

Histological analysis of the genito-urinary tract of 5 $RAR\gamma^{-/-}$ males at day 18.5 p.c. or 3 weeks p.p. failed to reveal any abnormality. However, $RAR\gamma^{-/-}$ males which survived to sexual maturity (8 weeks or older) never impregnated females. Subsequent analysis showed that in all cases (8 out of 8), the seminal vesicles and prostate glands exhibited squamous metaplasia and/or keratinization of the glandular epithelia (Fig. 4, compare panels d-g (WT controls) with panels h-l (mutants)). The mucosal folds and septa characteristic of normal seminal vesicles and prostate are often completely missing in $RAR\gamma^{-/-}$ mutants (panels h, j and l; compare to wild type glands in panels d and f) and in some cases, the two glands cannot be distinguished from one another on the basis of histological criteria (panel j). The glandular epithelia (GE, panels e and g) are replaced by a squamous stratified epithelia of either the non-keratinizing (SQ, panels h and i) or of the keratinizing (SQK, panels j to l) type. The lumens of the glands contain little or no secretion product (S in panels d and f; compare to panels h, j and l). On a macroscopic scale, one can note: (i) an atrophy of the seminal vesicles (SV; compare $RAR\gamma^{-/-}$ and WT SV in panel a; note the loss of the characteristic white coloration of the functional SV of normal, sexually mature, animals; see also panel b); (ii) an atrophy of the cranial prostate (CP; compare $RAR\gamma^{-/-}$ CP in panel b to WT CP in panel a; note that the SV and CP in panel b are indistinguishable); (iii) in some cases, hypertrophy of the cranial prostate (compare the CP on the right of panel c to the contralateral gland); this hypertrophy is probably generated by an infection of the gland causing focal destruction of the metaplastic epithelium accompanied by foreign body reactions to the retained keratinizing cells (see panel l). Interestingly, RNase protection analysis using tissues from WT animals revealed that $RAR\gamma 1$ was the major RAR transcript expressed in the seminal vesicle and prostate glands

- 45 -

of mature males, whereas $RAR\alpha$ and β RNAs were barely detectable under conditions where their expression was readily revealed in tissues such as vas deferens, testes and lung (data not shown). No genito-urinary defects were seen in $RAR\gamma^{-/-}$ females, which were fertile.

5

Resistance to retinoic acid treatment

In the mouse, exposure to RA at day 8.5-9.0 p.c. has been shown to cause, among other abnormalities, craniofacial malformations, homeotic transformations of the axial skeleton, and spina bifida with complete truncation of the caudal axial skeleton (for references see Description Of The Related Art and, Kessel and Gruss, *Cell* 67:89-104 (1991); Kessel, M., *Development* 115:487-501 (1992); see also Fig. 5d and h). To determine if any of these malformations could be mediated by $RAR\gamma$, pregnant dams from heterozygote crosses were treated with RA at day 8.5 or 9.0 p.c., and we examined day 18.5 p.c. fetuses for skeletal malformations. Strikingly, none of the $RAR\gamma^{-/-}$ embryos exposed to RA at day 8.5 p.c. developed spina bifida, truncation of the axial skeleton in the post-thoracic region, degenerate or fused ribs, disorganized vertebral centres or malformed neural arches in the lower thoracic-lumbar-sacral region (Fig. 5b and f, Table III). All WT fetuses exhibited these malformations as previously described (Kessel, M., *Development* 115:487-501 (1992); Fig. 5d and h, Table III). Interestingly, $RAR\gamma$ heterozygotes were partially resistant to these RA-induced truncations (compare Fig. 5c to d and g to h; see Table III), suggesting that a critical level of $RAR\gamma$ must be present for RA to fully induce these defects. $RAR\gamma^{+/-}$ animals, however, always exhibited lumbo-sacral vertebral malformations, including fusion of the neural arches and disorganized vertebral centres (Fig. 5c and g, Table III). Under identical conditions, $RAR\gamma^{2-/-}$ animals exhibited the same type and frequency of RA-induced malformations as control littermates (data not shown).

As expected from previous studies (Kessel, M., *Development* 115:487-501 (1992)), treatment of WT embryos with RA at day 9.0 p.c. led to

- 46 -

progressively more posterior truncations and associated defects when compared to treatment at day 8.5 p.c. (Table III, and data not shown). Again, these malformations were never observed in $RAR\gamma^{-/-}$ littermates (Table III). In contrast, $RAR\gamma$ was apparently not involved in RA-induced craniofacial malformations or thoracic homeotic transformations, e.g. anterior transformations of the eighth thoracic vertebra (Table III, Fig. 5f). Strikingly, lumbar to thoracic, sacral to lumbar and lumbar to sacral vertebral transformations also occurred in RA-treated $RAR\gamma$ null homozygotes (Fig. 5f, Table III, and data not shown). Similar transformations were previously seen by Kessel and Gruss, *Cell* 67:89-104 (1991) in WT animals treated with a lower dose of RA.

Table III

Comparison of RA-Induced Skeletal Malformations In Wild Type, $RAR\gamma^{+/+}$ And $RAR\gamma^{-/-}$ Offspring

		RA treatment at day 8.5 p.c.			RA treatment at day 9.0 p.c.		
		RAR γ genotype			RAR γ genotype		
		+/+	+/-	-/-	+/+	+/-	-/-
5	Number of fetuses examined at day 18.5 p.c.	11	16	10	13	24	18
	Malformations						
	Craniofacial malformations	11 (100%)	16 (100%)	10 (100%)	13 (100%)	24 (100%)	18 (100%)
	Homeotic transformations:						
	- 8 vertebrosteral ribs (T8→T7)	7 (64%)	8 (50%)	6 (50%)	7 (54%)	9 (38%)	11 (61%)
10	- 14 or 15 ribs (L→T)	6 (54%)	14 (87%)	7 (70%)	7 (54%)	8 (33%)	11 (61%)
	- Number of lumbar vertebrae: 5	NA	NA	1 (10%)	NA	NA	1 (7%)
	6	NA	NA	9 (90%)	NA	NA	15 (83%)
	7	NA	NA	0 (0%)	NA	NA	3 (16%)
	Caudal truncations	11 (100%)	13 (81%)	0 (0%)	13 (100%)	14 (58%)	0 (0%)
15	Malformed lumbar/sacral vertebrae	NA	3 (19%)	0 (0%)	NA	10 (42%)	0 (0%)
	Degenerate ribs	10 (91%)	6 (38%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Table III. Comparison of RA-induced skeletal malformations in wild type, $RAR\gamma^{+/-}$ and $RAR\gamma^{-/-}$ offspring. Genotypes were derived from analysis of DNA from the placenta of day 18.5 p.c. fetuses from dams that had received a single dose of RA (100 mg/kg) at either day 8.5 or 9.0 p.c. Skeletons were prepared, and malformations scored under a dissecting microscope. Where possible, fetuses from the same litter were compared. NA; not applicable.

Discussion

The results clearly demonstrate that the expression of at least one functional $RAR\gamma$ isoform is essential for normal development and physiology of the mouse. The results also show that $RAR\gamma$ is directly involved in the transduction of the retinoic acid signal in the animal, since some of the abnormalities seen in the $RAR\gamma$ null mutant mice have been previously described in animals fed a vitamin A deficient (VAD) diet, and some of the teratogenic effects of maternal RA administration do not occur in $RAR\gamma$ null fetuses.

Abnormalities in $RAR\gamma$ null mice and in animals fed a VAD diet.

Retardation of growth, emaciation, atrophy of certain organs and gross changes occurring in the eye are the main macroscopic alterations which are characteristic of animals fed a VAD diet (Wolbach, S.B., and Howe, P.R., *J. Exp. Med.* 42:753-777 (1925); for reviews and refs, see Sporn *et al.*, *The retinoids*, Vols. 1 and 2, Sporn *et al.*, eds., Academic Press, Orlando, Florida (1984); Livrea and Packer, in *Retinoids*, Livrea and Packer, eds., Marcel Dekker, New York (1993)). These animals eventually die, with no obvious lethal lesions. The viability of $RAR\gamma$ null homozygotes is similarly impaired and no distinctive lethal lesions could be identified. Interestingly, there is a marked variability in early lethality of $RAR\gamma$ deficient animals, which appears

to be correlated with growth-deficiency. A similar observation has been made in the case of RAR α null animals. Whether this reflects concerted effects of RAR α and RAR γ on the same processes whose failure leads to early death, or whether RAR α and RAR γ act independently on different pathways whose impairment can independently lead to death, is unknown.

Wide-spread squamous metaplasia and/or keratinizing squamous metaplasia of various epithelia is a landmark of vitamin A deprivation (Wolbach, S.B., and Howe, P.R., *J. Exp. Med.* 42:753-777 (1925); Underwood, J.N., "Vitamin A in animal and human nutrition," in *The retinoids*, Vol. 1, Sporn, M.B., *et al.*, eds. (Academic Press, Inc., Orlando, FL), pp. 282-392 (1984)). This occurs in the respiratory tract (in the nares, as well as in the larynx, trachea and bronchi), in the alimentary tract (submaxillary glands, parotid gland, accessory salivary glands of the tongue and pharynx, and pancreatic ducts), in the genito-urinary tract (bladder, renal pelvis, uterus and oviducts, epididymis, prostate, seminal vesicles), and eyes and related glands (conjunctiva, Meibomian gland ducts, cornea, intra and extra-orbital lacrimal glands, and Harderian glands). It was found that the seminal vesicles and prostate glands of RAR γ null homozygote mature males exhibit a similar keratinizing squamous metaplasia to that described in VAD, and in which it can be reversed by RA. (Wolbach, S.B., and Howe, P.R., *J. Exp. Med.* 42:753-777 (1925); Dowling and Wald, *Proc. Natl. Acad. Sci. USA* 46:587-608 (1960); Howell *et al.*, *J. Reprod. Fertil.* 5:159-167 (1963)). These epithelial transformations most probably account for the sterility of RAR γ deficient males. In marked contrast, all other epithelia which exhibit squamous metaplasia in VAD animals (see above) appear normal in RAR γ null animals, suggesting that the other RARs, or RXRs, can substitute for RAR γ in the retinoid maintenance of these unaffected tissues. This possibility is supported by the observation that RAR γ 1 transcripts are the only RAR transcripts which we could detect in prostate and seminal vesicles.

Approximately 75 % of the offspring of VAD females exhibit a broad array of congenital malformations which affect, among others, the eye, genito-urinary tract (e.g. agenesis of the seminal vesicles and prostate), aortic arches,

heart, lungs, diaphragm, kidneys, and the genital ducts (Wilson, J.G., *et al.*, *Am. J. Anat.* 92:189-217 (1953)), none of which were seen in day 18.5 p.c. RAR γ null homozygotes. Interestingly, squamous metaplasia, but not agenesis of the seminal vesicle and prostate glands occurred in RAR γ ^{-/-} males, implicating a different RAR type in development of these organs. RAR γ deficient fetuses did, however, exhibit two congenital malformations (apart from axial skeleton abnormalities). The agenesis of the Harderian gland, in which retinoic acid preferentially accumulates (Sharma *et al.*, "Comparative distribution of retinol and all-trans retinoic acid in pregnant hamsters," in *Retinoids*, Livrea and Packer, eds., Marcel Dekker, New York, pp. 569-584 (1993)), is particularly remarkable in view of its great variability, since not all animals were similarly affected. The lack of keratinizing squamous metaplasia in the Harderian glands of RAR γ null homozygotes (although it is frequently observed in VAD animals; see above), indicates that RAR γ is specifically involved in Harderian gland organogenesis, but not in the maintenance of its glandular epithelium. The second congenital malformation, that of tracheal cartilaginous rings, is also remarkable, since it is the only malformation present in all RAR γ null fetuses at day 18.5 p.c., and also because this cartilage is the only one affected even though RAR γ is expressed in all cartilages during organogenesis (Ruberte *et al.*, *Development* 108:213-222 (1990)). Hence, if RAR γ has a general function in cartilage formation as suggested (Ruberte *et al.*, *Development* 108:213-222 (1990)), the other RARs (or RXRs) must be able to fulfill this role in all other cartilages (e.g. larynx and bronchi). This suggests a specific role for RAR γ in the development of the tracheal cartilaginous rings, since RAR α transcripts are also found in the mesenchyme of the developing trachea (Dollé *et al.*, *Development* 110:1133-1151 (1990)).

Approximately 50% of the RAR γ null newborns are selectively cannibalized within 24 hours by their mothers. Hence, a fraction of these newborns must exhibit some abnormality that not yet detected, but is clearly recognized by mice. This phenotype was not scored in offspring from VAD dams.

Axial specification in RAR γ null homozygotes

Gain-of-function (Kessel *et al.*, *Cell* 61:301-308 (1990); Lufkin *et al.*, *Nature* 359:835-841 (1992)) and loss-of-function (Le Mouellic *et al.*, *Cell* 69:251-264 (1992)) studies have shown that some Hox genes specify the identity of occipital bones and vertebrae. Although there are noteworthy exceptions (Pollock *et al.*, *Cell* 71:911-923 (1992); Jegalian and De Robertis, *Cell* 71:901-910 (1992)), gain-of-function mutations lead to posteriorizations, while loss-of-function mutations lead to anteriorizations. That Hox gene expression could be controlled during development by retinoic acid has been suggested by the observation that some Hox gene transcripts accumulate in cultured embryonal carcinoma (EC) cells exposed to RA (most notably those of the most 3' Hox paralogues, e.g. Hoxa-1; for refs see Simeone *et al.*, *Nature* 346:763-766 (1990); Simeone *et al.*, *Mech. Dev.* 33:215-228 (1991); LaRosa and Gudas, *Proc. Natl. Acad. Sci. USA* 85:329-333 (1988); Papalopulu *et al.*, *Nuc. Acids. Res.* 19:5497-5506 (1991)). Furthermore, it has been shown that RA administration at various times during gestation results in both changes in the expression of some Hox genes along the anterioposterior axis (Morriss-Kay *et al.*, *EMBO J.* 10:2985-2995 (1991); Conlon and Rossant, *Development* 116:357-368 (1992); Marshall *et al.*, *Nature* 360:737-741 (1992)) and anterior or posterior homeotic transformations of vertebrae, depending on the precise time of RA administration and the vertebral region which is affected (Kessel and Gruss, *Cell* 67:89-104 (1991); Kessel, M., *Development* 115:487-501 (1992)).

Loss of RAR γ leads to homeotic transformations (mainly anteriorizations) which occur with variable frequencies along the anterioposterior axis, demonstrating that its presence, and presumably also RA, is required for proper specification of some cervical and thoracic vertebrae. Whether this specification is directly exerted through Hox genes remains to be established by *in situ* hybridization analysis. Interestingly, Hoxb-4 (Hox-2.6) gene knock-outs also result in an identical axis to atlas anterior transformation. Moreover, Hoxb-4 expression is altered in embryos from RA-treated dams

- 52 -

(Conlon and Rossant, *Development* 116:357-368 (1992)) and Hoxd-4 (Hox-4.2), a paralogue of Hoxb-4, has been shown to contain a RA-response element (Pöpperl and Featherstone, *Mol. Cell. Biol.* 13:257-265 (1993)). It is, however, unclear at which stage of vertebral specification RAR γ could be involved. RAR γ transcripts have been detected posterior to the caudal neuropore in late gastrulating embryos in the mesoderm and overlying neuroectoderm where somites have not yet differentiated. RAR γ expression then apparently disappears concomitant with the appearance of somites (Ruberte *et al.*, *Development* 108:213-222 (1990)). These *in situ* hybridization observations clearly suggest that RAR γ plays a role in somite formation and specification at a time where RA administration is known to affect both Hox gene expression and axial specification (Kessel and Gruss, *Cell* 67:89-104 (1991)). RAR γ could also be involved in the specification of vertebral identity at a later stage (day 10.5 p.c.), when RAR γ transcripts are found in sclerotomies. Such a possibility is supported by the observation that exposure of embryos to RA at this stage can lead to "respecification" of vertebral identities (Kessel, M., *Development* 115:487-501 (1992)). However, this late "respecification" effect could not be correlated with altered Hox gene expression, hence suggesting that RAR γ may also be involved in the maintenance of vertebral identities through a mechanism not involving Hox genes. Note that homeotic transformations have not been found in fetuses from VAD dams, perhaps because it may necessitate a vitamin A deprivation too drastic to allow gestation to proceed (see Wilson, J.G., *et al.*, *Am. J. Anat.* 92:189-217 (1953)). That the homeotic transformations described here reflect a true function of RAR γ , rather than that of another closely linked gene that may have been affected during gene targeting, is supported by a marked increase in the frequency of their occurrence in mice lacking both RAR γ and RAR α 1 genes.

The absence of lumbosacral truncations in RAR γ null fetuses treated with RA at days 8.5 - 9.0 p.c., demonstrates that at least some of the functions of the various RARs can be specific. Similar resistance to RA-induced malformations was not observed in RAR α 1 null homozygotes, even

though this receptor is co-expressed with RAR γ in the caudal presomitic mesoderm at this developmental stage (Ruberte *et al.*, *Development* 108:213-222 (1990)). Paradoxically, further studies have indicated that RAR γ 2 is the major RAR γ isoform expressed at this stage in the caudal presomitic mesoderm (C. Wolf, Ph. K. and P.C., unpublished results), yet RAR γ 2 null homozygotes display the same frequency of RA-induced malformations as their WT and heterozygote littermates. Clearly, a lower level of expression of RAR γ 1 (relative to RAR γ 2) is sufficient to mediate the lumbosacral truncations which lead to spina bifida. Interestingly, RA administration is still able to induce vertebral homeotic transformations in the lumbosacral and more rostral regions of the axial skeleton of RAR γ null homozygotes, thus indicating that these transformations are not mediated by RAR γ . The lack of defects in the lumbosacral region of untreated RAR γ null homozygotes implies that the RAR γ -mediated RA-induced malformations are unlikely to reflect the perturbation of a "normal" function of RAR γ , but rather a pharmacological effect with no physiological counterpart. Hence, care should be taken in extrapolating from teratogenic effects to the physiological functions of RA.

It has been recently reported that Hoxa-1 (Hox 1.6) contains a RA response element (Langston and Gudas, *Mech. Dev.* 38:217-228 (1992)) and Hoxa-1 RNA is induced by RA (LaRosa and Gudas, *Proc. Natl. Acad. Sci. USA* 85:329-333 (1988); Simeone *et al.*, *Mech. Dev.* 33:215-228 (1991)). Knock-outs of the Hoxa-1 gene have generated specific alterations (e.g. of the inner ear and of acoustico-facial nerves and ganglia), in the region which corresponds to its rostral domain of expression (Lufkin *et al.*, *Cell* 66:1105-1119 (1991); Chisaka *et al.*, *Nature* 355:516-520 (1992)). No similar alterations were seen in either RAR γ or RAR α null homozygotes indicating that either Hoxa-1 expression is not RA-dependent, or that the different RARs are functionally redundant for Hoxa-1 gene expression. A similar redundancy may account, at least in part, for the lack of significant changes in the levels of the RA-responsive RAR isoforms RAR α 2 (Leroy *et al.*, *Proc. Natl. Acad. Sci. USA* 88:10138-10142 (1991)) and RAR β 2 (Zelent, A., *et al.*, *EMBO J.*

10:71-81 (1991); Mendelsohn *et al.*, *Development* 113:723-734 (1991); and refs therein) in RAR γ null homozygotes (Fig. 1d) or RAR α null homozygotes.

It is widely assumed that RA plays a critical role in limb pattern formation, possibly as a morphogen (reviewed in Tabin, C.J., *Cell* 66:199-217 (1991)). This assumption has been supported by the demonstration that a topical application of RA can trigger the expression of the Hox genes which are thought to be instrumental in specifying the antero-posterior axis of the limb (reviewed in Duboule, D., *BioEssays* 14:375-384 (1992)). It is remarkable that RAR γ null mutants do not display any limb malformations, even though RAR γ is uniformly expressed in the limb bud at the time of morphogenesis, and its expression becomes selectively restricted to precartilaginous condensations at later stages (Dollé *et al.*, *Nature* 342:702-705 (1989); Ruberte *et al.*, *Development* 108:213-222 (1990)). Since RAR α (but not RAR β) is ubiquitously expressed in the limbs at these stages, RAR γ and RAR α may fully functionally overlap, since limb defects were also not observed in RAR α null homozygotes. Double null mutants for both RAR α and RAR γ have to be generated to further investigate the actual role of RA in normal limb development.

Functional redundancy in the RA receptor multigene family and variations in mutation penetrance and expressivity.

It has been previously shown that RAR γ transcripts, which are first detected at day 8.0 p.c. in all three germ layers in the presumptive posterior region of the embryo, are then found between days 9.5 and 11.5 p.c. uniformly distributed in the mesenchyme of the frontonasal region, pharyngeal arches, limb buds and sclerotomes (Ruberte *et al.*, *Development* 108:213-222 (1990)). At day 12.5 p.c., RAR γ transcripts are found in all precartilaginous mesenchymal condensations as well as in the genital tubercle mesenchyme. From day 13.5 p.c., RAR γ transcripts become specifically restricted to all cartilages, irrespective of whether they will subsequently ossify or not, and to all differentiating keratinizing squamous epithelia (skin, oral cavity,

- 55 -

oesophagus, left wall of the stomach), as well as to developing teeth and whisker follicles. At day 14.5 p.c. and later, RAR γ transcripts are found in all three skin layers (Dollé *et al.*, *Development* 110:1133-1151 (1990); Ruberte *et al.*, *Development* 108:213-222 (1990)). The present study clearly establishes that the presence of RAR γ is not critically required for most of these tissues to be formed or maintained. With the exception of abnormalities of cervical and thoracic vertebrae, no other skeletal malformations were found in RAR γ null mutants. Similarly, the tracheal cartilage was the only cartilage to be malformed, and all keratinizing squamous epithelia, including skin, were normal in RAR γ null mutants. The absence of malformations in tissues and organs derived from the mesenchyme of the branchial arches and the genital tubercle is also noteworthy.

These apparently discordant observations indicate that RAR γ transcripts are expressed in many places in the embryo and in the adult animal where the RAR γ protein is either never synthesized or, if synthesized, is not playing any critical role. Alternatively, or additionally, there could be a considerable functional overlap between members of the RA receptor family. Indeed functional redundancy appears to be a recurrent theme among members of multiple gene families (reviewed in Tautz, D., *BioEssays* 14:263-266 (1992); Brookfield, J., *Evolutionary Genet.* 2:553-554 (1992)). Since RAR α (essentially RAR α 1) appears to be ubiquitously expressed, it could possibly substitute for most RAR γ functions, the exceptions corresponding to the observed defects. These may correspond to either subsets of genes whose expression is specifically controlled by RAR γ , or to cells which express predominantly RAR γ . The absence of any detectable abnormalities in RAR γ 2 null mutant mice represents an extreme case of redundancy, where all RAR γ 2 functions would be apparently fulfilled by RAR γ 1 or another RAR. Note, however, that the evolutionary conservation of the RAR γ 2 isoform from fish to man, both in sequence and expression pattern (Ellinger-Ziegelbauer and Dreyer, *Genes and Dev.* 5:94-104 (1991)) necessarily implies that it performs a specific function. This function may be difficult to uncover, since it may require examination of RAR γ 2 null animals in the wild and/or a study of a

- 56 -

large number of animals: to have a 95 % chance of finding significant evidence for a 5 % viability disadvantage (which is significant over evolutionary times) would require examination of more than 20,000 individuals (Kimura, M., *Genetics* 47:713-719 (1962); Brookfield, J., *Evolutionary Genet.* 2:553-554 (1992)).

An extensive functional redundancy amongst RARs should in fact not be too surprising, since with the exception of the N-terminal A/B regions, all three RARs and their isoforms are still very similar in their amino acid sequence (Leid *et al.*, *TIBS* 17:427-433 (1992)), and hence may still perform a number of common regulatory functions, albeit possibly with different efficiencies. Variations in these efficiencies, which may be affected by the genetic background, could account for the variable penetrance of most of the abnormalities seen in the RAR γ null mutants. The hypothesis that functional redundancy between RAR α and RAR γ may be at least partially responsible for the observed variations in the penetrance of most of the RAR γ null mutant defects is supported by preliminary results obtained with mice lacking both RAR γ and RAR α 1. In contrast to RAR γ null homozygotes, all RAR γ null/RAR α 1 null double homozygotes present a complete bilateral agenesis of the Harderian glands. In this respect, it is important to note that RAR α 1 null homozygotes do not display any abnormal phenotype.

The variability, within a given animal, in the expressivity of a number of abnormalities of the RAR γ null mutant phenotype (eg. Harderian glands) obviously cannot be ascribed to variations in genetic background. However, this variability is also likely to be related, at least in part, to functional redundancy between the various RARs, as exemplified by the complete bilateral agenesis of the Harderian glands in all RAR α 1 null/ RAR γ null double homozygotes. Hence, the variations in expressivity of the RAR γ null mutation in this bilateral structure most probably reflect the stochastic nature of gene activity in single cells (reviewed in Ko, M.S.H., *BioEssays* 14:341-346 (1992)), which may itself be dependent on variations in RAR α 1 cellular levels and/or in regulatory factors which synergize with RAR α 1. Similar stochastic variations may account for the occurrence of unilateral homeotic

- 57 -

transformations in the cervical and thoracic regions (Fig. 2 and Table II), and more generally of any asymmetrical malformation of bilateral or symmetrical structures.

Making additional mouse mutants lacking more than one receptor type or isoform should reveal the extent of redundancy between RA receptors, and the actual specific physiological functional role played by each RAR and RXR in retinoid signalling *in vivo*.

Example 2

Function of Retinoic Acid Receptor α (RAR α) in the Mouse

High Postnatal Lethality and Testis Degeneration in Retinoic Acid Receptor α (RAR α) Mutant Mice

To investigate the function of RAR α in the mouse, either the whole gene or specifically the RAR α 1 isoform has been knocked-out. A disruption of the whole gene leads to high postnatal lethality and testis degeneration with no other detectable developmental or postnatal abnormalities, suggesting that besides its requirement for the maintenance of spermatogenesis, the function of RAR α is critical only for some homeostatic processes. Interestingly, RAR α 1 null mutant mice had no detectable phenotype, which suggests a surprising redundancy between RAR isoforms.

Experimental Procedures

RAR α Homologous Recombination

Mutant alleles of the RAR α gene were created by homologous recombination using the "replacement" vector strategy (M.R. Capecchi, *Science* 244:1288 (1989)) (Fig. 6a). For the RAR α full disruption, the construct was prepared as follows. The 23 kilobase pair (kb) SalI insert from

- 58 -

the phage λ G2mRAR α (Leroy *et al.*, *EMBO J.* 10:59 (1991)) was partially end-filled and subcloned into the partially end-filled BamHI site of pBluescriptSK+ to create plasmid p231. The 11 kb EcoRI-SpeI fragment (containing the A2 and B regions of RAR α) from p231 was subcloned into the
5 EcoRI-XbaI sites of pTZ18R to create plasmid p807. Site-directed mutagenesis (using an oligo with 20 nucleotide flanking arms) was performed on single-strand DNA from p807 to insert a seven nucleotide mutation (TGAGCGG) after the CCA encoding the proline at amino acid 19 of the B region (Leroy
10 *et al.*, *EMBO J.* 10:59 (1991)) creating an in-frame stop (TGA) and a unique NotI restriction site to generate plasmid p819, into which the 1.7 kb NotI fragment containing the GTI-II enhancer-driven neomycin gene (purified from p581, Lufkin *et al.*, *Cell* 66:1105 (1991)) was cloned to generate p826B1, which was linearized at the unique SalI site and used for electroporation. p826B1 has 8 kb of homologous genomic DNA sequence 5', and 3 kb of
15 homologous sequence 3' to the NotI site of the neomycin insertion. For the RAR α 1 isoform disruption, a 9 kb EcoRV-SalI fragment from λ G1mRAR α (Leroy *et al.*, *EMBO J.* 10:59 (1991)) was subcloned into pBluescript+ to generate pD182, which was partially digested at the KpnI site (at amino acid 19 of the A1 region) into which was subcloned the 1.4 kb KpnI enhancer-less
20 RSV-TATA box-driven neomycin gene fragment (derived from p581, Lufkin *et al.*, *Cell* 66:1105 (1991)) to generate pD183, which was subsequently digested with EcoRV and ligated with the 2.3 kb GTI-II enhancer-driven HSV-tk gene fragment [purified from p565 (Lufkin *et al.*, *Cell* 66:1105 (1991)) by digesting with EcoRV] to generate plasmid pD209 which was linearized at the
25 unique SpeI site and used for electroporation into D3 embryonic stem cells (Gossler *et al.*, *Proc. Natl. Acad. Sci. USA* 83:9065 (1986); Lufkin *et al.*, *Cell* 66:1105 (1991)). pD209 contains 8.0kb and 1.0kb of homologous DNA sequence 5' and 3' respectively, to the site of neomycin insertion. Genomic DNA extraction, Southern blotting, ES cell culture, generation of chimeras,
30 and probe preparation were as described (Lufkin *et al.*, *Cell* 66:1105 (1991)). Probes 1, 2 and 3 correspond to a 1.4 kb XbaI-SpeI fragment from p231, a 0.8 kb BamHI fragment from λ G1 mRAR α , and a 1.4 kb NotI-SmaI fragment

- 59 -

from λ G1 mRAR α , respectively. Both probes 2 and 3 were used in the case of the RAR α 1 disruption.

Two types of mutant alleles were generated. The first mutant allele (termed RAR α) prevents the synthesis of all isoforms of RAR α by disruption of exon 8 which encodes the receptor region B common to all isoforms. The second mutant allele (termed RAR α 1) selectively prevents the synthesis of the RAR α 1 isoform by disruption of exon 3 which encodes the RAR α 1-specific region A1. Following electroporation into D3 embryonic stem (ES) cells (Gossler *et al.*, *Proc. Natl. Acad. Sci. USA* 83:9065 (1986)) and selection in G418 (or G418 and gancyclovir in the case of the RAR α 1 disruption), resistant ES clones were expanded and analyzed by Southern blotting for a homologous recombination event (data not shown, see Fig. 6a and b). The RAR α and RAR α 1 constructs gave 7 and 3 homologous recombination events per 32 and 22 resistant colonies, respectively. 5 and 3 positive ES clones for RAR α and RAR α 1 mutations were injected into 890 and 498 blastocysts, which resulted in 25 and 23 male chimeric animals, respectively. One of the RAR α ES clones (KC25) and 3 of the RAR α 1 ES clones (KA3, KA5, and KA26) gave germ-line transmission. The heterozygous mice for either mutation appeared healthy, normal, and were fertile. Intercrossing of heterozygous mice for either mutation produced homozygous offspring (Fig. 1b, and below).

RNase Protection Analysis

To verify that the RNAs encoding the RAR α and RAR α 1 proteins were disrupted, a RNase protection assay was performed using RNA from day 13.5 p.c. embryos (a time at which RAR α RNA is abundantly expressed, see Ruberte *et al.*, *Development* 111:45 (1991)) (Fig. 6c). RNA was purified by the single-step guanidinium-isothiocyanate-phenol technique as described (P. Chomczynski and N. Sacchi, *Analytical Biochem.* 162:156 (1987)) with the addition of a phenol-chloroform extraction step. 50 μ g of embryo RNA was used per hybridization reaction which was at 55°C for 8-12 hrs. The

- 60 -

conditions for probe preparation and RNase protection were essentially as described (Sambrook *et al.*, eds., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1987)). Conditions for transcribing with T7 RNA polymerase was as recommended by the supplier (Promega Biotec). Plasmid constructions (used as templates to transcribe antisense [³²P]-labelled RNA probes) were as follows. For RNase protection of RAR α in the RAR α gene disruption, the 384 bp KpnI-PstI RAR α cDNA fragment (Leroy *et al.*, *EMBO J.* 10:59 (1991)) spanning the A1-C region was cloned into the KpnI-PstI site of pTZ19U to generate plasmid p971, which was linearized at the unique XmnI site and transcribed with T7 polymerase; for RAR α in the RAR α 1 disruption, the 361 bp Ball-AccI fragment from the RAR α 1 cDNA (Leroy *et al.*, *EMBO J.* 10:59 (1991)) was subcloned into pBluescriptSK+ which was linearized at the XhoI site and transcribed with T7 polymerase; for CRABPI, the 418 bp EcoRI-AvaI cDNA fragment (C.M. Stoner and L.J. Gudas, *Cancer Research* 49:1497 (1989)) was end-filled with Klenow enzyme at the AvaI site prior to digestion with EcoRI, and then subcloned into the EcoRI-SmaI site of pTZ19U to generate plasmid p948 which was linearized at the unique Scal site and transcribed with T7 polymerase; the plasmid for the CRABPII RNase protection contains the 830 bp EcoRI-HindIII CRABPII cDNA (Giguère *et al.*, *Proc. Natl. Acad. Sci. USA* 87:6233 (1990)) fragment cloned into the EcoRI-HindIII sites of pBluescriptSK+ which was linearized at the unique Scal site and transcribed with T7 polymerase; for mouse Histone H4, the 630 bp EcoRI-HindIII Histone H4 genomic fragment was subcloned into the EcoRI-HindIII site of pTZ19U to generate p323 which was linearized at the unique NaeI site and transcribed with T7 polymerase; for RAR γ , nucleotides 235-600 of the RAR γ 1 cDNA spanning the A1-C regions were amplified by PCR with oligos containing BamHI and EcoRI sites at the end, the PCR fragment was digested with BamHI and EcoRI and subcloned into the BamHI-EcoRI site of pBluescriptSK+, which was linearized with BamHI and transcribed with T7 polymerase; the plasmid for RAR β RNase protection was created as follows: the 334 bp PstI RAR β 2 cDNA fragment spanning the A2-C regions was

- 61 -

subcloned into the PstI site of pBluescriptSK+, linearized with BamHI and transcribed with T7 polymerase. The sizes of the protected fragments are as indicated.

Results

5 *Expression of RAR α Isoforms*

As expected, wild type embryos and embryos heterozygote for the RAR α mutation expressed the two major RAR α isoforms (RAR α 1 and RAR α 2; Fig. 6c, lanes 2 and 3). However, in the RAR α null homozygotes, only the mutant form of the RAR α RNA was present (RAR α mut; Fig. 6c, compare lane 4 with lanes 2 and 3). Similarly, for the RAR α 1 mutation, wild type and heterozygote embryos expressed wild type RAR α 1 and α 2 RNAs (Fig. 6c, lanes 5 and 6, and data not shown), but only the mutant form of the RAR α 1 RNA (RAR α 1 mut) and the RAR α 2 RNA were detectable in the RAR α 1 homozygotes (Fig. 6c, compare lane 7 with lanes 5 and 6, and data not shown). Examination of the RNA levels of the two other RARs (RAR β and RAR γ) showed no significant variation between wild type, heterozygote or homozygote embryos for either mutation (Fig. 6c), indicating that globally RAR α does not play a unique role in controlling RAR β and RAR γ whose expression has been shown to be enhanced by retinoic acid (Mendelsohn *et al.*, *Development* 113:723 (1991); Lehmann *et al.*, *Mol. Cell. Biol.* 12:2976 (1992); and refs therein). Verification that no RAR α protein was present in the RAR α homozygotes was achieved by western blot analysis of nuclear proteins isolated from day 13.5 p.c. RAR α wild type, heterozygote, and homozygote embryos (Fig. 1d). For Western blot analysis, the embryo was thawed on ice, transferred to 3ml of lysis buffer (20mM KCl, 10mM Tris-HCl pH 7.0, 1mM MgCl₂, 1.5mM EDTA, 10% glycerol, 1mM PMSF) and dounced with 20-30 strokes of an "A" pestle. Complete cellular lysis and liberation of nuclei was verified by microscope analysis of an aliquot. Nuclei were spun out at 2000g for 5 minutes at 4°C. The cytosolic supernatant was removed and stored at

- 62 -

-80°C. Nuclei were subsequently extracted in lysis buffer containing 0.6M KCl and 25% glycerol. Debris was removed by spinning at 105,000 g for 1 hr. Protein concentration was determined by a colorimetric assay (Bio-Rad). Whole cell extracts from Cos-1 transfected cells were prepared as described (Rochette-Egly *et al.*, *J. Cell Biol.* 115:535 (1991)). Sample denaturation, electrophoresis, transfer to nitrocellulose, blocking, and antibody probing were as described (Rochette-Egly *et al.*, *J. Cell Biol.* 115:535 (1991)). Detection of the primary antibody was performed with Protein-A coupled horseradish peroxidase binding followed by chemiluminescence detection as described by the manufacturer (Amersham). Rabbit polyclonal antibodies specific to RAR α [RP α (F)] and RAR β [RP β (F)2] were generated as described (Gaub *et al.*, *Experimental Cell Res.* 201:335 (1992); Rochette-Egly *et al.*, *Mol. Endocrinology* 6:2197 (1992)).

Using antibodies directed against the C-terminal F region of RAR α , the RAR α protein was readily observed in wild type and heterozygote RAR α embryo extracts (Fig. 6d, lanes 3 and 4), whereas no specific RAR α protein was detected in RAR α homozygotes (Fig. 6d, lanes 5 and 6). In agreement with the results of RNA analysis, immunoblotting with antibodies directed against the F region common to all RAR β isoforms did not reveal any significant variation (within the sensitivity of the assay) between the same protein extracts (compare Fig. 6d, lanes 11-14).

Viability of RAR α and RAR α 1 Null Homozygotes

The viability of RAR α and RAR α 1 null homozygotes was determined by intercrossing heterozygous animals and analyzing the distribution of offspring at various times during gestation and following birth. RAR α 1 null homozygotes represented ~25% of the offsprings at all gestational and post-natal stages (Table 4, and data not shown). Furthermore, RAR α 1 homozygotes were fertile and intercrossing of homozygotes generated litters of RAR α 1 null animals which appear healthy, fertile, and phenotypically normal. Histological analysis and wholemount skeletal staining did not reveal any detectable

- 63 -

malformations in the $RAR\alpha$ 1 null homozygotes. When analyzed during gestation or caesarian-delivered at day 18.5 p.c., $RAR\alpha$ null homozygotes also represented ~25% of all embryos and fetuses, demonstrating that full disruption of the $RAR\alpha$ gene is not embryonic lethal. No obvious malformations or lesions could be macroscopically or histologically detected. However, genotyping of animals as soon as 12-24 hours post-partum (p.p.) showed a 60% deficiency of $RAR\alpha$ null homozygotes. Yet all caesarian-delivered animals survived up to 24 hrs when isolated from their dams, indicating that up to 60% of the homozygotes had been preferentially cannibalized by their mother during this brief period (see Table IV, 1 day p.p.). Analysis at increasingly later times showed a continuing decrease in $RAR\alpha$ null homozygotes relative to wild type and heterozygote littermates, with homozygotes representing only 3% of the total population at 1-2 months of age (Table IV). In fact, 75% of the $RAR\alpha$ null homozygotes which remain after one day will disappear during the following 1-2 months. Some of these animals showed a slower growth rate after 1-2 weeks, and before death became emaciated and lethargic. No obvious malformations or lesions could be detected, with the exception that 60% of these homozygotes displayed webbed digits on both forelimbs and hindlimbs; however the precise digits fused varied between individuals and between limbs within the same animal (Figure 7). This interdigital webbing never regressed and persisted until the death of the animal. Alizarin red/alcian blue staining of bone and cartilage showed that the webbed phenotype was restricted to soft tissues (Fig.7). This phenotype was not seen in wild type or $RAR\alpha$ heterozygous animals for which the digits become fully separated by 2 weeks of age.

The small number of remaining $RAR\alpha$ homozygotes which survived for more than 2 months surprisingly appeared superficially normal, being of similar size as their wild type or heterozygote littermates, but none of the five males tested (up to the age of 5 months) sired any offspring, even though caged with fertile wild type females. Examination of the testes of four of these males at the age of 4-5 months showed severe degenerative changes of the germinal epithelium (Fig. 8). The parenchyma of the testes of $RAR\alpha$ null

homozygotes displayed patchy lesions of the seminiferous tubules, with rare tubules which appeared histologically normal (T1, Fig. 8b and f; compare with 2a and d), while adjacent tubules were markedly atrophic (T3, Fig. 8c) and/or mostly devoid of spermatogenic cells (e.g. spermatogonia, spermatocytes, spermatids and spermatozooids; see T2 in Fig. 8b and c, and Fig. 8e). In addition, vacuolation was frequently seen within the cytoplasm of Sertoli cells (v, compare Fig. 8b and c with Fig. 8a, and Fig. 8e with Fig. 8d), and cytoplasmic expansions of these cells often partially filled the lumen of the seminiferous tubules (T2, see Fig. 8b, c and e). The lumen of the epididymal duct of $RAR\alpha$ null homozygotes contained very few spermatozooids (Z, compare Fig. 8g and h). Thus, spermatogenesis appeared to be drastically reduced in the testes of $RAR\alpha$ null homozygotes, although it was not totally abolished as indicated by the presence of a few spermatozooids in the seminiferous epithelium of rare tubules and in the lumen of the epididymal duct. In contrast no lesions were observed in the seminal vesicles and prostate (not shown).

Involvement of $RAR\alpha$ in the Maintenance of Homeostatic Processes

Animals fed a vitamin A-deficient (VAD) diet develop a syndrome, which includes among others the following symptoms: widespread substitution of keratinizing squamous epithelium for normal epithelium, atrophy of several glandular organs, eye lesions, testis degeneration, and emaciation (S.B. Wolbach and P.R. Howe, *J. Exp. Med.* 42:753 (1925); B. Underwood, in *The Retinoids I*, Sporn, *et al.*, eds., Academic Press, Inc., Orlando, FL, pp. 282-392 (1984); Thompson *et al.*, *Proc. Royal Society* 159:510 (1964)). These animals eventually die. In addition, offspring of VAD females exhibit a broad array of abnormalities which mainly involve the eye, the genito-urinary tract, the kidney, the heart, and the lung (Wilson *et al.*, *Am. J. Anat.* 92:189 (1953)). The present results demonstrate that $RAR\alpha$ plays a crucial role in transducing the retinoid signal in the animal, since ~90% of the $RAR\alpha$ null homozygotes die before the age of two months. As is the case for the VAD

- 65 -

syndrome, some of these animals have a slower rate of growth and become emaciated, even though no specific lethal lesions can be identified macroscopically or histologically. Thus, RAR α appears to be involved in the maintenance of some homeostatic processes, as has been previously inferred from its apparently ubiquitous expression in the adult animal (M. Leid *et al.*, *Trends in Biochem. Sci.* 17:427 (1992); Leroy *et al.*, *EMBO J.* 10:59 (1991); Dollé *et al.*, *Development* 110:1133 (1990); Ruberte *et al.*, *Development* 111:45 (1991)). Surprisingly, with the exception of testis degeneration, RAR α null homozygotes do not display any of the VAD-associated lesions, and histomorphogenesis is apparently normal. These observations suggest that the other RARs and/or RXRs (M. Leid *et al.*, *Trends in Biochem. Sci.* 17:427 (1992) for refs) may mediate the retinoid signal in the events which are reflected by the occurrence of specific VAD developmental abnormalities and post-natal lesions, or alternatively, that the other RARs and RXRs can substitute for RAR α in the retinoid control of these events. Note, however, with respect to this possible redundancy, that the domains of expression of the other RAR and RXR transcripts generally appear to be more restricted than that of RAR α (M. Leid *et al.*, *Trends in Biochem. Sci.* 17:427 (1992); Leroy *et al.*, *EMBO J.* 10:59 (1991); Dollé *et al.*, *Development* 110:1133 (1990); Ruberte *et al.*, *Development* 111:45 (1991); Dollé *et al.*, *Nature* 342:702 (1989); Mangelsdorf *et al.*, *Genes & Develop.* 6:329 (1992)). This suggests that either the expression domains of the other RARs are wider than revealed by *in situ* hybridization, or that in many locations, transcription of the RAR α gene does not reflect an actual function of the receptor.

The Role of RA in Spermatogenesis

It has been claimed that vitamin-A (ROL) deficiency leads to testis degeneration which cannot be reversed by RA administration, implying that ROL plays a unique role not only in vision, but also in spermatogenesis (Thompson *et al.*, *Proc. Royal Society* 159:510 (1964); McHowell *et al.*, *J. Reprod. Fertil.* 5:159 (1963)). The present study shows that the presence of

5 RAR α , and therefore most probably of RA, is required for maintenance of a functional germ line epithelium in adult males. Interestingly, the degeneration of the germinal epithelium in RAR α null homozygotes is similar, if not identical, to that observed in males maintained on a VAD diet (McHowell *et al.*, *J. Reprod. Fertil.* 5:159 (1963); Ismail *et al.*, *Am. J. Anat.* 188:57 (1990); and refs therein). Thus, the results strongly suggest that it is RA, and not ROL, which is required for the maintenance of spermatogenesis. This conclusion is also supported by recent observations which have shown that the repeated administration of high doses of RA can in fact restore spermatogenesis in males fed a VAD diet (H.M.M. Van Pelt, and D.G. De Rooij, *Endocrinol.* 128:697 (1991)). The presence of CRABPI and RAR α (Porter *et al.*, *J. Androl.* 6:197 (1985); Eskild *et al.*, *Biol. Reprod.* 44:55 (1991); K.H. Kim and M.D. Griswold, *Mol. Endocrinol.* 4:1679 (1990)) in germ cells further supports the view that RA may be the active retinoid in spermatogenesis. As previously suggested the requirement for ROL may reflect the existence of a blood-testis barrier preventing RA to reach the adluminal compartment of the seminiferous tubules (Porter *et al.*, *J. Androl.* 6:197 (1985); Shingleton *et al.*, *Biochemistry* 28:9641 (1989)). The CRBPI-containing Sertoli cells which form this blood-testis barrier may normally convert ROL to RA to subsequently deliver it to the germ cells (Porter *et al.*, *J. Androl.* 6:197 (1985); Shingleton *et al.*, *Biochemistry* 28:9641 (1989)). Interestingly, it has been proposed (Porter *et al.*, *J. Androl.* 6:197 (1985)) that the blood-testis barrier is less restrictive in birds where the typical mammalian Sertoli-Sertoli cell junctions are absent and spermatogenesis can be restored by RA in ROL-deficient animals (Thompson *et al.*, *Br. J. Nutr.* 23:471 (1969)).

RAR α Null Homozygote Phenotype

30 The selective cannibalism of RAR α null homozygote newborns indicate that they exhibit an abnormal phenotype recognized by their mothers. Interestingly, not all null homozygote newborns are eaten, which suggests that

the "cannibalizable" phenotype has a variable penetrance, which may be related to the non-homogeneous genetic background of the null homozygote newborns. Also, the variable penetrance of the webbed digit phenotype, which appears to be associated with early death (~2-3 weeks), and the longer survival of a small fraction of RAR α null homozygotes (>2 months), may have a similar origin. Moreover, it is noteworthy that the webbing is often different when comparing pairs of limbs of a given animal. This variability, which cannot be accounted for by variation in the genetic background, is most probably related to the stochastic nature of gene activity (reviewed in M.S.H. Ko, *BioEssays* 14:341 (1992)) in the cells which give rise to bilateral and symmetrical structures within an animal.

The transcripts of RAR α 1, which is the most abundant RAR α isoform, are ubiquitously expressed, whereas those of RAR α 2, which is the second most common RAR α isoform, could not be detected by *in situ* hybridization (Leroy *et al.*, *EMBO J.* 10:59 (1991); Leroy *et al.*, *Proc. Natl. Acad. Sci. USA* 88:10138 (1991)). It is therefore surprising that, in agreement with a recent report (Li *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:1590 (1993)), RAR α 1 null homozygotes did not exhibit any of the abnormalities seen in RAR α null animals. This may mean that RAR α 1 and RAR α 2 are essentially functionally redundant, and that RAR α 2 may have a wider domain of expression than suggested from the *in situ* hybridization data (note, however, that the global expression of RAR α 2 was not altered in RAR α 1 null homozygotes). Alternatively, in most places RAR α 1 transcription may not reflect an actual function of this isoform, and RAR α 2 may fulfil most of the function of the RAR α gene. In any event, the high degree of conservation of RAR α 1 across vertebrates indicates that this isoform must perform some specific function conferring a selective advantage (see D. Tautz, *BioEssays* 14:263 (1992); J. Brookfield, *Evolutionary Genet.* 2:553 (1992)) not yet detected.

The almost ubiquitous expression of RAR α (mainly RAR α 1) has suggested that it may mediate the RA induction of the RA-responsive RARs, i.e. RAR α 2, RAR β 2, RAR γ 2 (M. Leid *et al.*, *Trends in Biochem. Sci.* 17:427 (1992); Leroy *et al.*, *Proc. Natl. Acad. Sci. USA* 88:10138 (1991);

Mendelsohn *et al.*, *Development* 113:723 (1991); Lehmann *et al.*, *Mol. Cell. Biol.* 12:2976 (1992); and refs therein). No change in the level of their expression was seen in RAR α (for RAR β 2 and RAR γ 2) or RAR α 1 null homozygotes, indicating that if RAR α is involved in these inductions, its function must be redundant. Note also that the level of expression of the RA-responsive CRABP II gene (Giguère *et al.*, *Proc. Natl. Acad. Sci. USA* 87:6233 (1990); Durand *et al.*, *Cell* 71:73 (1992)) was unchanged in RAR α null homozygotes (Fig. 1c). RAR α is also the only RAR whose expression could be detected in the precise rhombencephalic region (see M. Leid *et al.*, *Trends in Biochem. Sci.* 17:427 (1992) for a review) where the product of the RA-inducible homeogene *Hoxa-1* (*Hox-1.6*) (A.W. Langston and L. Gudas, *Mechanisms of Development* 38:217 (1992)) is known to play a critical role during morphogenesis (Lufkin *et al.*, *Cell* 66:1105 (1991); Chisaka *et al.*, *Nature* 355:516 (1992)). Interestingly, no hindbrain or inner ear lesions resembling those resulting from *Hoxa-1* knock-outs (Lufkin *et al.*, *Cell* 66:1105 (1991); Chisaka *et al.*, *Nature* 355:516 (1992); and M.M., T.L. and P.C., unpublished data) were seen in RAR α null homozygotes. Therefore, it appears that either *Hoxa-1* expression is not critically dependent on RA-induction in the animal, or that other RARs or RXRs whose expression has not been detected by *in situ* hybridization in this region of the hindbrain could control the RA-responsiveness of *Hoxa-1*.

- 69 -

Table IV**Viability of $RAR\alpha^{-/-}$ and $RAR\alpha^{+/-}$ Offspring**

	$RAR\alpha^{+/-} \times$	$RAR\alpha^{+/-}$	offspring
	$-/-$	$+/-$	$+/+$
Gestation day 8.5 to 18.5	34 (0.9)	64 (1.6)	39 (1.0)
1 day p.p.	13 (0.4)	56 (1.6)	36 (1.0)
5 2 weeks old	15 (0.2)	123 (1.9)	64 (1.0)
1-2 months	4 (0.1)	90 (2.0)	45 (1.0)
	$RAR\alpha^{+/-} \times$	$RAR\alpha^{+/-}$	offspring
	$-/-$	$+/-$	$+/+$
1-2 months	52 (0.9)	108 (1.9)	58 (1.0)

The genotypes of offspring from intermatings of either $RAR\alpha$ or $RAR\alpha 1$ heterozygote animals are given. Different litters were genotyped at the time shown on the left. Hence, one can only compare horizontal rows of numbers. At each time point, the distribution of offspring of the different genotypes is shown in parentheses relative to wild type ($+/+$). Note that the percentage of $RAR\alpha$ null homozygotes decreases with time. This decrease plateaus at 1-2 months with only ~10% of the $RAR\alpha$ homozygotes still alive.

Example 3**Function of Retinoic Acid Receptor $\beta 2$ ($RAR\beta 2$)
in the Mouse****Experimental Procedures****Disruption of the $RAR\beta 2$ Locus**

The isolation of genomic clones containing the first exon encoding the m $RAR\beta 2$ isoform (exon 4, E4, Fig. 9a) was as described (Zelent *et al.*,

- 70 -

EMBO J. 10:71-81 (1991)). The 6.5kb and the 3'-adjacent 3.7kb KpnI fragments containing the RAR β 2 first exon and intronic DNA, respectively, were subcloned into the KpnI site of the pTZ19r vector (Promega). For construction of the targeting vector the XhoI/KpnI 5.5 kb fragment containing the mRAR β 2 first exon (Fig. 9a) was excised from the pTZ19r vector and subcloned into the pBluescript SK(-) vector (Stratagene). The XhoI site was subsequently destroyed, and a new XhoI site was introduced into the mRAR β 2 first exon (exon 4) by site-directed mutagenesis with the oligonucleotide 5'-GATCATGTTTGACTGACTCGAGTGGATGTTCTGTCAG-3' spanning nucleotides 459-491 (Zelent *et al.*, *EMBO J.* 10:71-81 (1991)); in this way both of the potential initiation codons (nucleotide positions 463 and 475, Zelent *et al.*, *EMBO J.* 10:71-81 (1991)) were destroyed. Following introduction of the XhoI site, the 3.7 kb intronic KpnI fragment (Fig. 9a) was introduced 3' to the 5.5 kb fragment. The GTI-II enhancer-driven neomycin gene (NEO cassette, derived from the p566 vector, Lufkin *et al.*, *Cell* 66:1105-1119 (1991)) was then introduced into the newly created XhoI site of the 5.5 kb (formerly XhoI/KpnI) fragment (Fig. 9a). Finally the 2.3 kb GTI-II enhancer-driven herpes simplex virus thymidine kinase gene fragment (purified from plasmid p565, Lufkin *et al.*, *Cell* 66:1105-1119 (1991)) was inserted into the pBluescript SK(-) Sall site to yield the mRAR β 2-targeting vector (mRAR β 2- Δ). For electroporation, mRAR β 2- Δ was linearized at its unique NotI site (Fig. 9a). Genomic DNA extraction, Southern blotting, embryonic stem (ES) cell culture, generation of chimeras and probe preparation were as described elsewhere (Lufkin *et al.*, *Cell* 66:1105-1119 (1991)). Probe 1 was the KpnI/XhoI fragment derived from the 5' end of the 6.5 kb KpnI fragment containing the mRAR β 2 first exon.

RNA Analysis

Total RNA was prepared from 13.5 dpc embryos using the single-step guanidinium-isothiocyanate-phenol technique (Chomczynski *et al.*, *Anal. Biochem.* 162:156-159 (1987)). RT-PCR was performed according to Huet

- 71 -

et al., *Development* 118:613-627 (1993). Reactions were carried out in 100mM Tris-HCl, pH 8.8, 500mM KCl, 14mM MgCl₂ using 1μg of RNA per reaction in a final volume of 50μl. Primer oligonucleotides were as indicated in figure legends. After denaturation at 94°C for 3 min and annealing for 10 min at 50°C, Taq polymerase and AMV reverse transcriptase were added, and incubation was continued for 15 min at 50°C. PCR amplification was then performed (denaturation 30 sec at 94°C, annealing 30 sec at 55°C, and extension 1 min at 72°C) for 20 or 30 cycles. The reaction mixtures were electrophoresed on 2% agarose gels, and then transferred to Hybond N filters. Hybridization was carried out with RAR isoform-specific cDNA fragments or oligonucleotide probes. For RNase protection assays, 30μg of RNA was used per hybridization reaction. The conditions for preparation of the probes and RNase protection were as described in *Current Protocols in Molecular Biology*, J. Wiley Interscience, New York, eds. Ausubel *et al.* (1987).

Protein Analysis

Embryos from RARβ2 heterozygote intercrosses were collected at 14.5 dpc by caesarian section, and genotyped. Nuclear extracts were prepared from wild type, heterozygous and homozygous mutant RARβ2 embryos. Whole cell extracts from transfected COS-1 cells and nuclear extracts from embryos were prepared as described (Gaub *et al.*, *Exp. Cell Research* 201:335-346 (1992); Rochette-Egly *et al.*, *J. Cell Biol.* 115:535-545 (1991)). Western blotting and immunodetection procedures were also as described (Rochette-Egly *et al.*, *J. Cell Biol.* 115:535-545 (1991)), using as antibody preparations rabbit polyclonal antisera specific for RARα ((RPα(F), Gaub *et al.*, *Exp. Cell Research* 201:335-346 (1992)), and RARβ (RPβ(F)2, Rochette-Egly *et al.*, *Molecular Endocrinology* 6:2197-2209 (1992)), and a monoclonal antibody (ascites) directed against the RARβ2 A2-region (Rochette-Egly *et al.*, *Molecular Endocrinology* 6:2197-2209 (1992)). Immunoreactions were visualized using protein A-coupled to horseradish peroxidase, followed by chemiluminescence (Amersham).

Animal Mating and RA Treatment

Timed matings were performed by placing mice in the reversed light-cycle for 3 weeks, followed by placement of males and females together for 3 hours at the midpoint of the dark cycle. Evidence of mating was determined by the presence of a vaginal plug and was considered to be time 0 dpc. For all other matings males and females were left together for the night and the presence of a vaginal plug in the morning was taken as 0.5 dpc. To generate $RAR\beta 2^{-/-}$ mutants expressing the $RAR\beta 2/lacZ$ transgene (Mendelsohn *et al.*, *Development* 113:723-734 (1991)), $RAR\beta 2/lacZ^{+/+}$ homozygote mice were mated with $RAR\beta 2^{-/-}$ mutants to generate $RAR\beta 2^{+/-}/RAR\beta 2/lacZ^{+/-}$ animals. In a second round of mating $RAR\beta 2^{+/-}/RAR\beta 2/lacZ^{+/-}$ mice were backcrossed with $RAR\beta 2/lacZ^{+/+}$ transgenic mice to generate males with the genotype $RAR\beta 2^{+/-}/RAR\beta 2/LacZ^{+/+}$. To analyze the expression of $RAR\beta 2/lacZ$ promoter activity in $RAR\beta 2^{-/-}$ mutant embryos, $RAR\beta 2^{+/-}/RAR\beta 2/LacZ^{+/+}$ males were mated with $RAR\beta 2^{+/-}$ females producing offspring which all contained the transgene, thus allowing comparisons of promoter activity within a given litter containing wild type, heterozygous and homozygous mutant $RAR\beta 2$ embryos.

For generation of hindbrain malformations timed mating experiments were performed and all-trans RA (Sigma) (25mg/kg body weight) was administered to the mother by oral gavage at 7.25 dpc. Embryos were collected at 9.0 dpc, yolk sacs were taken for genotyping and embryos were fixed in 4% paraformaldehyde and embedded in paraffin for *in situ* hybridization on sections. For each embryo, three sets of alternate sections were hybridized to Hoxb-1, Krox-20 and Hoxa-3 probes. These probes have been described previously (Gaunt *et al.*, *Development* 104 suppl.:71-82 (1988); Wilkinson *et al.*, *Nature* 337:461-464 (1989); Hunt *et al.*, *Nature* 353:861-864 (1991)). No significant alteration in the Hoxa-3 expression pattern was observed in RA-treated embryos (data not shown). A separate series of embryos was processed for whole-mount *in situ* hybridization with a digoxigenin-labelled Hoxb-1 riboprobe. For induction of limb

- 73 -

malformations, all-trans RA was administered at 11.5 dpc (80mg/kg) and fetuses were collected at 18.5 dpc and analyzed for skeletal malformations. For investigating induction of RAR β 2 promoter activity in RAR β 2 mutants, 25mg/kg RA was administered at days 10.5 and 11.5 pc, then embryos were collected and stained for LacZ activity after 4 hours. For whole-mount skeletal analysis, fetuses were collected at 18.5 dpc by cesarean section and skeletons were prepared as described (Lufkin *et al.*, *Nature* 359:835-841 (1992)). Histological analysis was as in Lohnes *et al.* (*Cell* 73:643-658 (1993)) and lacZ staining as in Mendelsohn *et al.* (*Development* 113:723-734 (1991)).

Inactivation of the RAR β 2 Isoform

In order to inactivate the RAR β 2 isoform, the neomycin-resistance cassette (NEO) was inserted into the RAR β exon 4 (the RAR β 2 first exon), which encodes the 5'-untranslated (UT) and A2-region sequences (see Fig. 9a). Prior to insertion of the NEO cassette, the first two potential in-frame initiation codons of the RAR β 2 protein were destroyed by site-specific mutagenesis of the region spanning nucleotides 459-491 (see Zelent *et al.*, *EMBO J.* 10:71-81 (1991), and Materials and Methods), while a XhoI site was created to facilitate introduction of the cassette. The targeting construct contained approximately 3 kb of 5' and 6 kb of 3' homologous sequences (Fig. 9a). A cassette containing the herpes simplex virus thymidine kinase gene (HSV-TK) was inserted at the 5' end of the targeting construct in order to allow the use of the positive-negative selection technique (Thomas *et al.*, *Cell* 51:503-512 (1987)) to isolate ES cell clones which have undergone homologous recombination.

The RAR β 2 targeting vector was linearized and electroporated into D3 ES cells (Lufkin *et al.*, *Cell* 66:1105-1119 (1991)). Selection was performed with G418 and gancyclovir. Resistant colonies were expanded and analyzed by Southern hybridization for the presence of the correct targeting event employing a probe derived from DNA sequences located 5' to the border of

- 74 -

those contained in the targeting construct (probe 1, Fig. 9a). Three ES cell clones out of 90 analyzed yielded, in addition to the wild type 6.5 kb KpnI fragment, the 4.3 kb KpnI fragment expected for the disrupted *RAR β 2* allele (Fig. 9a and b; clones BH1, BH45 and BH32). To confirm that these ES cell clones had the correct targeting event, additional Southern hybridization experiments were performed (Fig. 9b). DNAs were digested with KpnI, BamHI and XbaI, and hybridized with either probe 1, a full-length neomycin gene probe or a probe derived from sequences located at the 3' end of the construct (data not shown). The 3 ES clones yielded the appropriate sized restriction fragments in all digests performed (Fig. 9a and b). In addition, Southern blots hybridized with the neomycin gene probe showed the restriction enzyme fragments expected for a single targeting event, indicating that no non-homologous recombination events had occurred in the ES cells which harbored the targeted allele (see Fig. 9b).

Results

RAR β 2 Mutant Mice Have a Normal Phenotype

Chimeric mice were generated from the targeted ES cell lines, and all transmitted the disrupted *RAR β 2* allele to their offspring. *RAR β 2*^{-/-} homozygous offspring were generated at the expected Mendelian frequency from intercrosses of *RAR β 2*^{+/-} heterozygotes (Fig. 10). *RAR β 2*^{-/-} mutants derived from either ES cell line were as fertile as wild type animals, lived as long as their wild type littermates and were apparently normal based on their external appearance. Histological analysis was also performed on two 18.5 day post coitum (dpc) fetuses which were serially sectioned. No malformations were detected. Further analysis of the internal organs from *RAR β 2*^{-/-} adults also did not reveal any abnormalities. *RAR β 2*^{-/-} animals were also examined for possible skeletal and cartilage anomalies using alizarin red and alcian blue double staining, but again, there were no obvious malformations (not shown).

- 75 -

Since $RAR\beta 2^{-/-}$ mutants were apparently normal, it was important to verify that no $RAR\beta 2$ protein was made. It was also important to rule out the possibility that a downstream initiation event might generate a truncated $RAR\beta 2$ protein, since there is a methionine codon embedded in a favorable Kozak sequence located between the two zinc fingers of the $RAR\beta 2$ C-region (Met 113, see Zelent *et al.*, *EMBO J.* 10:71-81 (1991)). Translational initiation from this site would result in a protein of ~ 39 kilodaltons, lacking the A and B regions as well as the first zinc finger of the DNA binding domain. Nuclear extracts were prepared from 14.5 dpc wild type embryos and from embryos heterozygous and homozygous for the $RAR\beta 2$ mutant allele. Extracts containing similar amounts of nuclear proteins were first immunoblotted with a rabbit polyclonal antibody specific for the F-region of $RAR\alpha$. The 51 kd $RAR\alpha 1$ protein was detected at equivalent levels in wild type, heterozygote and homozygote mutant embryo extracts (Fig. 11). The presence of the $RAR\beta 2$ protein was determined by immunoprecipitation with a monoclonal antibody specific for the $RAR\beta 2$ A2-region, followed by Western blot analysis using a polyclonal antibody directed against the F-region of $RAR\beta$. $RAR\beta +/+$ wild type extracts contained the 51kd $RAR\beta 2$ protein (Fig. 11), while extracts derived from $RAR\beta +/-$ heterozygous embryos retained 30-50% of wild type levels. Extracts prepared from $RAR\beta 2^{-/-}$ homozygotes, however, contained no detectable $RAR\beta 2$ protein. Since the A2-region-specific antibody used in the immunoprecipitation experiments shown in Fig. 11 would not react with a truncated $RAR\beta 2$ proteins lacking this amino-terminal sequence, additional immunoblotting experiments were performed using an antibody specific for the $RAR\beta$ F-region. Neither the putative 39kd truncated $RAR\beta 2$ protein (see above) nor any other sign of immunoreactivity could be detected in $RAR\beta 2^{-/-}$ extracts (data not shown), indicating that more C-terminally located methionine codons were not utilized as translational initiation sites in the mutant animals.

To further demonstrate that no wild type $RAR\beta 2$ protein could be produced in homozygote mutant embryos, it was determined whether wild type $RAR\beta 2$ RNA was synthesized in these mutants. Total RNA was prepared

- 76 -

from 13.5 dpc wild type, heterozygous and homozygous mutant embryos, and RNAs were identified employing the sensitive polymerase chain reaction coupled to reverse transcription (RT-PCR). Following RT-PCR, cDNA products were subjected to Southern blot analysis employing oligonucleotide probes which distinguish between the different RAR isoforms. To insure that the three RNA preparations were comparable, a cDNA fragment corresponding to the RAR α 1 isoform was amplified, employing a 5' primer located in the RAR α 1 A1-region (primer 5, Fig. 12a; see Leroy *et al.*, *EMBO J.* 10:56-69 (1991a)), and a 3' primer located in the RAR α C-region (primer 6, Figure. 12a). Equivalent amounts of the expected 338 nt-long RAR α 1 product were found irrespective of whether RNAs were prepared from RAR β 2+/+, RAR β 2+/- and RAR β 2-/- embryos (Fig. 12a). A second RT-PCR reaction was performed employing a 5' primer located in the RAR β 2 first exon (E4, in Fig. 9a) downstream from the site of insertion of the NEO cassette in the RAR β 2 gene (primer 2, Fig. 12a), and a 3' primer located in the RAR β 2 C-region (primer 1, Fig. 12a). The expected 232nt-long product (Fig. 12a) was obtained at similar levels from all three RNA preparations, indicating that the RAR β 2 locus was similarly transcribed in wild type, heterozygote and homozygote mutant embryos. To distinguish between mutant and wild type RAR β 2 transcripts, RT-PCR was performed with a 5' primer (primer 3, Fig. 12a) derived from the RAR β 2 5'-untranslated region (UTR) located upstream from the site of insertion of the NEO cassette, and the same 3' primer (primer 1, Fig 12a). This pair of primers should yield a 367ntlong product from wild type RAR β 2 RNA, while in embryos harboring the mutant allele, the RT-PCR product would be about 1.9 kb in length due to the insertion of the NEO cassette. For RNA prepared from 13.5 dpc wild-type embryos, the 367nt product corresponding to the wild type RAR β 2 cDNA was readily detectable, while it was reduced by about 50% in heterozygous embryos, and not detectable in RAR β 2 mutant homozygotes (Fig 12a). Much longer exposure of the autoradiograph (not shown) indicated that there was no wild type RAR β 2 transcript in homozygote embryos. Note that as expected a weak hybridization signal was detected in the RAR β 2 null mutant RNA,

- 77 -

which corresponded to the 1.9 kb mutant transcript (not shown). Thus, the RAR β 2 homozygote mutants appear to be null for both RAR β 2 transcript and protein.

The Levels of RAR β 1/ β 3, RAR α 1/ α 2 and RAR γ 1/ γ 2 Transcripts are Unchanged in RAR β 2 Null Mutant Embryos.

The RAR β 1/ β 3 isoforms are generated by alternative splicing of a primary transcript initiating from the 5' distal promoter (P1) and continuing through the RAR β 2 first exon (exon 4, which is independently transcribed from the downstream promoter P2; see Fig. 9a). Since the RAR β 1/ β 3 and RAR β 2 transcripts overlap, it was important to demonstrate that expression of the RAR β 1/ β 3 isoforms was not perturbed in the RAR β 2 null mutants. RT-PCR was performed to assess the levels of RAR β 1/ β 3 transcripts in wild type, heterozygous and homozygous mutant embryos. A 5'-primer located in the RAR β 1/ β 3 A-region (primer 4, Fig. 12a), and a 3' primer located in the RAR β C-region (primer 1, Fig. 12a) were used to amplify the RAR β 1/ β 3 cDNAs. The expected 306nt-long product corresponding to the RAR β 1 isoform was detected at similar levels in RNAs from wild type, heterozygote and homozygote embryos; the 387nt-long RAR β 3 product was also detected at approximately the same levels (Fig. 12a, and data not shown). These results show that there was no appreciable change in the steady state levels of the RAR β 1/ β 3 transcripts in the RAR β 2 null mutants, and therefore that the presence of the NEO cassette did not affect the splicing of the RAR β 1/ β 3 primary transcript.

Overexpression of the other RARs could possibly account for the normal phenotype of the RAR β 2 null animals. RNase protection experiments were performed using riboprobes (depicted in Fig. 12b) to investigate whether RAR β 2 inactivation had an effect on the levels of the RA-inducible RAR α 2 and RAR γ 2 transcripts, and on the levels of the non-inducible RAR α 1 and RAR γ 1 transcripts. Using an antisense RAR α 2 RNA probe, similar levels of the 379nt-long protected product corresponding to the RAR α 2 transcripts were

- 78 -

detected in RNAs prepared from wild type, heterozygote or homozygote mutant 13.5 dpc embryos (Fig. 12b). Similar amounts of RAR α 1 transcripts were also detected in all three preparations (Fig. 12b), consistent with the results of the Western blotting and RT-PCR experiments described above (see Fig. 11 and 12a). Similarly, there was no observable change in the levels of the RNAs encoding the RAR γ 1 and γ 2 isoforms in the RAR β 2 null embryos (Fig. 12b). Together these findings demonstrate that RAR β 2 was not required for the expression of the other main RAR isoforms, and also that no global compensatory increase of any of these isoforms occurred.

RAR β 2 is Not Required for RAR β 2 Promoter Activity and RA-inducibility in Transgenic Mice

Since the RAR β 2 promoter is RA-inducible via a RARE, it was investigated whether the levels of activity of the RAR β 2 promoter would be affected in the absence of RAR β 2. RAR β 2 null animals were crossed with transgenic animals expressing an RAR β 2 promoter/lacZ reporter gene (Mendelsohn *et al.*, *Development* 113:723-734 (1991)). Progeny from these crosses were mated, and the resulting offspring were genotyped and stained for β -galactosidase (β -gal) activity at 9.5 dpc, 10.5 dpc, 11.5 dpc and 12.5 dpc. No differences were observed in the intensity nor in the pattern of β -gal activity in the null mutants compared to wild type embryos at the times tested (Fig. 13a, and data not shown). Therefore, if the activity of the RAR β 2 promoter is controlled by RA under physiological conditions, RAR β 2 is not mandatory for mediating this control.

It has been previously reported that the RAR β 2 promoter activity is dramatically induced (in transgenic animals) following exposure to RA *in utero* (Mendelsohn *et al.*, *Development* 113:723-734 (1991); Zimmer *et al.*, *Development* 116:977-983 (1992); Shen *et al.*, *Int. J. Dev. Biol.* 36:465-476 (1992)). This promoter activity was observed in ectopic locations (such as in the rostral hindbrain), and was also increased in regions where the promoter was already active in the absence of RA treatment, such as the spinal cord and

- 79 -

the apical ectodermal ridge and core mesenchyme of the limb buds. To determine whether the RAR β 2 isoform was required for mediating this up-regulation, RA treatments were performed on days 10.5 and 11.5 pc. Embryos were exposed to 25mg/kg all-trans RA by maternal gavage, collected after 4 hours, and stained for β -gal activity. 10.5 dpc wild type and homozygous null embryos exhibited comparable induction of RAR β 2 promoter activity (Fig. 13a-c). In the hindbrain, the rostral expression boundary of promoter activity shifted cranially, from the level of rhombomere 7 (arrowhead in panel a) to more rostral hindbrain, up to at least rhombomeres 4 (just rostral to the otocyst in panels b and c) and 1 (arrowheads in panels b and c), as well as in discrete regions of the midbrain and forebrain, as previously reported (Zimmer *et al.*, *Development* 116:977-983 (1992)). Note that promoter activity was lower in rhombomere 5 (otocyst level) than in rhombomere 4. LacZ expression was also induced in the limb buds, by RA treatment on 11.5 dpc, both in the apical ectodermal ridge and in the core mesenchyme of wild type, heterozygous and homozygous embryos (data not shown). Note also the increased activity of the RAR β 2 promoter in neural crest cells emigrating in the direction of the heart (Fig. 13b and c, arrows). Thus, the RA-inducibility of the RAR β 2 promoter was apparently not impaired in the absence of the RAR β 2 isoform.

RAR β 2 is Not Mandatory to Mediate the Teratogenic Effects of Retinoic Acid in the Hindbrain and in the Limbs

During neurulation, RA-treated embryos develop ill-defined rhombomeric sulci and boundaries, and exhibit a severe truncation of the forebrain (Morris-Kay *et al.*, *EMBO J.* 10:2985-2995 (1991); Conlon *et al.*, *Development* 116:357-368 (1992)). The specific effects of excess RA on hindbrain development has also been studied using various molecular markers available for this segmented region of the brain. Administration of RA to pregnant mice at 7.25 to 8.0 dpc results in subsequent alterations of the pre-otic hindbrain, which were interpreted as transformations of rhombomeres 2

and 3 (R2 and R3), either towards R4 and R5 identities, respectively (Marshall, *et al.*, *Nature* 360:737-741 (1993)) or towards an expanded R4 identity (Morris-Kay *et al.*, *EMBO J.* 10:2985-2995 (1991); Conlon *et al.*, *Development* 116:357-368 (1992)). These conclusions were essentially drawn

5 (i) from the pronounced rostral duplication or expansion of the Hoxb-1 rhombomeric expression domain which is normally restricted to R4 in the segmented hindbrain at 8.5-9.5 dpc, (ii) from alteration in the domains of expression of Krox-20 which is normally expressed in R3 and R5 at 8.5-9.5 dpc and is present only in a narrow band in R5 after RA-treatment, and (iii)
10 from a rostral expansion of the expression domain boundary of Hoxb-2, which normally lies at the R2/R3 boundary. RAR β 2 is an obvious candidate for mediating these specific effects of RA, since RA treatment results in an up-regulation of the RAR β 2 promoter, shifting its boundary of activity in the hindbrain from rhombomere 7 to more rostral regions (see above).

15 If RAR β 2 were required for mediating the effects of excess RA on hindbrain development, the above RA-induced alterations in normal hindbrain segmentation and/or specification should not occur in RAR β 2 null mutants. RAR β 2 +/- intercrosses were set up, and pregnant females were given 25mg/kg all-trans RA at 7.25 dpc, a dose which is known to produce
20 rhombomeric alterations. Severe truncations of the forebrain were observed at 9.0 - 9.5 dpc in wild-type, and interestingly also in RAR β 2 null RA-treated embryos (Fig. 14a, b, g and h, and data not shown). Hoxb-1 expression was analyzed by whole-mount *in situ* hybridization. A rostral expansion of the Hoxb-1 transcript domain was observed both in wild type (Fig. 14a and c) and
25 in RAR β 2 null mutants (Fig. 14b, d and e). Hoxb-1 transcripts extended more rostrally than the putative R3/R4 boundary, almost to the anterior extremity of the ventral neuroepithelium (see panel e). This rostral expansion of Hoxb-1 transcripts in both wild-type and RAR β null embryos was clearly confirmed by *in situ* hybridization on serial sections (Fig. 14g and h). Krox-20
30 transcripts were not detected in the putative R3 (Fig. 14g and h), although they were observed caudal to Hoxb-1 transcripts in the putative R5 (Fig. 14f). Hence, the results obtained with RAR β 2 null mutant embryos are similar to

- 81 -

those previously reported for wild-type embryos treated with RA at similar stages (Morris-Kay *et al.*, *EMBO J.* 10:2985-2995 (1991); Conlon *et al.*, *Development* 116:357-368 (1992)). In light of these results it can be concluded that RAR β 2 is not mandatory for the generation of the hindbrain malformations following exposure to excess RA.

RA treatments were also performed at 11.5 dpc to determine whether RAR β 2 is required to produce the RA-induced limb malformations which have been previously reported (Kochhar *et al.*, *Teratology* 7:289-295 (1973); Alles *et al.*, *Teratology* 40:163-171 (1989)). These malformations include truncations of the long bones as well as loss or fusions of digits. RAR β 2 +/- heterozygous crosses were performed, and 11.5 dpc embryos were exposed to 80mg/kg RA in utero. Fetuses were then collected at 18.5 dpc and stained with alizarin red and alcian blue to visualize skeletal and cartilagenous elements. Both wild type and RAR β 2 null fetuses exhibited severely malformed limbs compared to untreated animals (Fig. 15, and data not shown). There were no apparent differences between wild type and RAR β 2 null fetuses in either the nature or the extent of the malformations produced. In both cases RAR β 2+/+ and RAR β 2-/- animals had truncated long bones, as well as missing digits as a result of RA excess. Thus, the presence of RAR β 2 does not appear to be mandatory to generate RA-induced malformations in the limb.

Discussion

The studies presented here indicate that RAR β 2 is apparently dispensable for normal embryonic development and in post-natal life. These studies also show that RA-induced abnormalities are produced in the hindbrain and limbs of RAR β 2 null mutants to the same extent as in wild type animals, demonstrating that RAR β 2 is not mandatory to transduce the teratological RA signal in these structures.

Lack of Developmental and Adult Abnormalities in RAR β 2 Null Mutants

Animals lacking the RAR β 2 isoform exhibit an apparently normal phenotype at all stages of their life. This finding is reminiscent of recent studies showing that mice deficient for the RAR α 1 (Li *et al.*, *Proc. Natl. Acad. Sci. USA* 90:1590-1594 (1993); Lufkin *et al.*, *Proc. Natl. Acad. Sci. USA* 90:7225-7229 (1993)) or RAR γ 2 (Lohnes *et al.*, *Cell* 73:643-658 (1993)) isoforms are apparently indistinguishable from their wild type littermates. In contrast, animals lacking all of the RAR α or RAR γ isoforms were affected, exhibiting post-natal lethality and vitamin A deficiency (VAD)-like defects (Lohnes *et al.*, *Cell* 73:643-658 (1993); Lufkin *et al.*, *Proc. Natl. Acad. Sci. USA* 90:7225-7229 (1993)). In each of the RAR gene knockouts reported to date, it is probable that other RARs, which are temporally and spatially coexpressed with the disrupted RAR, fulfill some of the functions of the inactivated receptor(s); in other words there is some functional redundancy in the RAR family. However, since the various RAR isoforms are well conserved across vertebrate evolution, they must perform at least one specific function, not yet discovered, either because the proper conditions have not been employed to reveal it, or because this function gives a slight vital advantage that cannot be detected over a few generations. Recent *in situ* hybridization studies indicate that at least two RAR isoforms are in general expressed in a given tissue. The RAR α 1 isoform appears to be expressed essentially everywhere in the developing embryo, while the RAR β 1/ β 3 and RAR β 2 expression domains are partially overlapping. In contrast, the extent of overlap between the RAR β and RAR γ transcripts seems to be very limited, and their expression patterns are often complementary (Dollé *et al.*, *Nature* 342:702-705 (1989) and Dollé *et al.*, *Development* 110:1133-1151 (1990); Ruberte *et al.*, *Development* 108:213-222 (1990); Ruberte *et al.*, *Development* 111:45-60 (1991) and Ruberte *et al.*, *Development* 118:267-282 (1993)). Thus, there are at least 2 RAR candidates, which could functionally compensate for the lack of the RAR β 2 isoform, RAR α 1 and to a lesser degree

- 83 -

RAR β 1/ β 3. Supporting this possibility, double mutants which lack RAR α 1 in addition to RAR β 2 die at birth and are severely malformed.

The possibility that some RARs can operate interchangeably for a number of functions does not necessarily imply that they perform in an identical manner, exhibiting equal DNA binding affinities and transactivation properties for the same RA target genes. More likely, some subsets of target genes are activated preferentially by certain isoforms, but can also be activated by other isoforms, albeit less efficiently. However, for all that can be detected under the present conditions, this redundancy would be sufficient to preserve the realization of the normal cascade of RA-dependent events in RAR β 2 null mutant. Moreover, as shown by the present results, this functional "interchangeability" occurs in the absence of any global compensatory increase of the other RAR isoforms.

RAR β 2 is Not Mandatory for RA-inducibility of RAR α 2, RAR β 2 and RAR γ 2 Transcripts.

The findings that the RAR β 2, α 2 and γ 2 promoters are up-regulated upon RA administration to cells in culture, and that RAR β 2 transcripts (Osumi-Yamashita *et al.*, *Develop. Growth and Differ* 34(2):199-209 (1992); Harnish *et al.*, *Differentiation* 45:103-108 (1990) and Harnish *et al.*, *Developmental Dynamics* 149:239-246 (1992); Rowe *et al.*, *Development* 11:1007-1016 (1991b)) and promoter activity (Mendelsohn *et al.*, *Development* 113:723-734 (1991); Shen *et al.*, *Int. J. Dev. Biol.* 36:465-476 (1992); Zimmer *et al.*, *Development* 116:977-983 (1992)) are up-regulated in RA excess and down-regulated upon RA deprivation in mouse embryos (Ul-Haq *et al.*, *Proc. Natl. Acad. Sci. USA* 88:8272-8276 (1991)), led us to investigate whether RAR β 2 plays an autoregulatory role in maintaining or inducing its own RA-dependent promoter activity, as well as the activity of the RAR α 2 and RAR γ 2 promoters. There was no significant change in steady state transcript levels of RAR α 2, β 2 and γ 2 isoforms in RAR β 2 null mutants compared to wild type animals. In addition, it was found that the activity of

- 84 -

the RAR β 2 promoter fused to the lacZ reporter gene remained unchanged in transgenic animals lacking the RAR β 2 isoform. Furthermore, this reporter gene was induced to the same extent by RA excess in wild type and mutant animals. Taken together, these results indicate that RAR β 2 is not required to maintain or induce its own promoter activity nor is it required for the normal synthesis of RAR α 2, β 2 and γ 2 mRNAs. It also does not appear that the levels of RAR β 1/ β 3 transcripts are affected by the lack of RAR β 2, indicating that RAR β 2 is also not mandatory to mediate the RA-dependent relief of the elongation block which appears to preclude the expression of RAR β 1/ β 3 isoforms in the absence of RA. All of these results may indicate that RAR β 2 is never mediating the effect of RA on the expression of these various RA-responsive RAR isoforms, and that in its absence another RAR (e.g. RAR α 1) could substitute for it. Alternatively, under normal circumstances, RAR β 2 could preferentially mediate these RA responses. In this respect however, note that there was no observable global modification in the levels of RAR α 2, RAR β 2 and RAR γ 2 transcripts in RAR α null mutants (Li *et al.*, *Proc. Natl. Acad. Sci. USA* 90:1590-1594 (1993); Lufkin *et al.*, *Proc. Natl. Acad. Sci. USA* 90:7225-7229 (1993)).

RAR β 2 is Not Mandatory to Mediate the Teratogenic Effects of RA in the Hindbrain and in the Limbs

RA excess results in overexpression and/or ectopic expression of RAR β 2 transcripts at sites which later develop abnormally, such as the craniofacial region, the limbs, and the hindbrain (see above for refs), probably reflecting the presence of abnormally high levels of RA at these sites due to the condition of RA excess (Rossant *et al.*, *Genes Dev.* 5:133-1344 (1991); Balkan *et al.*, *Proc. Natl. Acad. Sci. USA* 89:3347-3351 (1992). These altered expressions of the RAR β 2 isoform raise the question as to whether this could be the cause of the teratological abnormalities resulting from RA excess. Wild-type and RAR β 2 null mutant embryos were challenged in utero with teratogenic doses of RA, and we compared the malformations produced in

- 85 -

both cases. In the hindbrain, the normal rostral boundary of RAR β 2 promoter activity is at the level of rhombomere 7, and following RA-treatment, this boundary is shifted to include more rostral rhombomeres, where alterations in rhombomeric segmentation and gene expression are also observed (see above for refs.). It is clearly demonstrated here that the presence of RAR β 2 isoform is not required to generate this shift in RAR β 2 promoter activity and the RA-induced morphological and molecular hindbrain alterations. Similar results were obtained when the limb malformations generated by RA treatment at 11.5 dpc in wild type and RAR β 2 mutant fetuses were examined. The same abnormalities were observed in both cases, including losses of digits and truncations of the radius and ulna. Thus, the results demonstrate that RAR β 2 is not mandatory for mediating the RA-induced teratogenic effects in hindbrain and limbs. This is in contrast with the specific requirement of RAR γ for the generation of truncations of the lumbo-sacral region by RA excess (Lohnes *et al.*, *Cell* 73:643-658 (1993)). Additional studies with other RAR single and double mutants will be necessary to discriminate between several possibilities, namely (i) that these teratological effects are never mediated by RAR β 2, (ii) that they are normally mediated by RAR β 2 which can be substituted by e.g. RAR α 1 in the absence of RAR β 2, and (iii) that several RARs can similarly mediate these effects.

Example 4

Function of Retinoic Acid Receptor RXR α in the Mouse

Experimental Procedures

Targeting Vectors and Homologous Recombination

Genomic clones for the mRXR α locus were obtained by screening a genomic library established in γ EMBL3 from D3 ES cell DNA, with a

- 86 -

mRXR α cDNA probe. Exon mapping of these clones showed that the RXR α gene extends over 30 kb and that its exonic organization is similar to that of the RXR γ gene (Liu and Linney, *Mol. Endocrinol.* 9:651-658 (1993)). In particular, most of the DNA binding domain is encoded in a single exon (exon 4; this exon contains the sequences corresponding to nucleotides 541 to 871 of the RXR α cDNA (numbering according to the genbank sequence No. M84817)). To construct the targeting vector, a 8 kb HindIII genomic fragment, containing exons 3 and 4, was first inserted into a plasmid harboring the GTI.11-tk cassette (Lufkin *et al.*, *Cell* 66:1105-1119 (1991)), yielding pHR(RXR α).1. Subsequently, a 1 kb EcoRI-XbaI fragment which contains exon 4 was removed and replaced with a PGK-NEO(A+) cassette derived from pKJ-1. This plasmid, pHR(RXR α).2, was linearized with NotI and electroporated into D3 or H1 ES cells as previously described (Lufkin *et al.*, *Cell* 66:1105-1119 (1991)). After selection with G418 and gancyclovir (Lufkin *et al.*, *Cell* 66:1105-1119 (1991)) resistant clones were expanded and analyzed by Southern Blotting.

Histological Procedures

Mouse embryos and fetuses were fixed in Bouin's fluid, processed for paraplast embedding, sectioned at 7 μ m and stained with hematoxylin and eosin or with Groat's hematoxylin and Mallory's trichrome (Mark *et al.*, *Development* 119:319-338 (1993)).

In Situ Hybridization Analysis

For *in situ* hybridization, embryos at 9.5 and 10.5 dpc were fixed in 4% paraformaldehyde and embedded in paraffin. All sections were collected on 4 or 5 sets of slides which were hybridized to α -cardiac actin, myosin heavy chain α (MHC α), myosin light chain 1 atrial (MLC1A), probes. At each developmental stage, heterozygous (+/-) and wild-type (WT; +/+) littermate embryos were processed in the same way for comparison. Probe

- 87 -

labelling, *in situ* hybridization and emulsion autoradiography were performed as described in (Decimo *et al.*, *In Gene Probes*, B.D. Hames, D. Higgins, eds., Volume II (1994)).

Three-Dimensional Computer Reconstruction

5 The same image processing configuration described by (Mark *et al.*, *Development* 119:319-338 (1993)) was employed.

Electron Microscopy

10 Two RXR α -/- and two WT fetuses (14.5 dpc) were fixed by intraventricular perfusion with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.05M cacodylate buffer (pH 7.2, 4°C). The eyes and the hearts were removed and immersed during 6 hours in the same fixative at 4°C. They were then washed overnight in cacodylate buffer. After post-fixation in 1% osmium tetroxide in cacodylate buffer for 1 hour at 4°C, they were dehydrated in graded series of ethanol and propylene oxide and embedded in Epon.

15 Semithin sections (2 μ m) were stained with toluidine blue for light microscopy. Ultrathin sections were contrasted as usual with uranyl acetate and lead citrate, and examined in a Phillips 301 electron microscope operating at 80 Kv.

Results

20 Generation of Mice and ES Cells Carrying Mutated RXR α Genes

 A replacement-type vector and positive-negative selection (Cappecci, *Science* 244:1288-1292 (1989)) was used to target the RXR α gene. In this vector, a 1kb EcoRI/XbaI fragment containing exon 4 (which encodes most of the RXR α DBD) was removed and replaced with a cassette containing the

25 neomycine phosphotransferase (NEO) gene and the PGK polyadenylation

sequences under the control of the PGK promoter (PGK-NEO(A+), Fig 16a). After electroporation into 129sv derived D3 or H1 ES cells and selection with G418 and gancyclovir, 3 resistant clones were positive for homologous recombination as judged from Southern Blot analysis of *SpeI* digested DNA with probe A (Fig 16a, and data not shown). These positive clones were further analyzed with an internal probe (probe B) and a NEO probe to confirm that the expected targeting event had occurred, and also to exclude random integrations of the vector or other rearrangements at the *RXR α* locus (data not shown). Two clones, FP104 (derived from D3 ES cells) and FM23 (derived from H1 ES cells), were injected into C57/Bl6 blastocysts and the resulting chimeric males were crossed with wild-type (WT) C57/Bl6 females. Chimeras derived from either of these two clones transmitted the mutation through their germline. Both lines of animals exhibited a similar phenotype, both at the heterozygote and homozygote stages (see below).

Heterozygous animals were fertile, and WT (+/+), heterozygous (+/-), and homozygous (-/-) embryos were obtained from crosses between heterozygous mice (see Fig 16b). Northern Blot analysis of total RNA extracted from 12.5 dpc embryos failed to detect *RXR α* transcripts in (-/-) embryos (Fig 16c). Therefore, even though the mutation could have conceptually generated *RXR α* transcripts that would just be lacking the sequences from exon 4, it appears that insertion of the PGK-NEO cassette in antisense orientation resulted in impaired transcription and/or processing of the *RXR α* RNA. Western blot analysis of nuclear extracts of 12.5 dpc embryos with an anti-*RXR α* antibody confirmed that no *RXR α* protein was synthesized in homozygous (-/-) embryos (Fig 16d). Thus the mutation that was generated is a null mutation for the *RXR α* gene.

In order to detect possible functional interactions between *RXR α* and RARs, *RXR α* +/- mice were crossed with mice heterozygous for a null allele of the *RAR γ* or *RAR α* genes (Lohnes *et al.*, *Cell* 73:643-658 (1993); Lufkin *et al.*, *Cell* 66:1105-1119 (1991)). These breedings generated fertile double heterozygous *RXR α* +/- *RAR α* +/- and *RXR α* +/- *RAR γ* +/- animals which

- 89 -

were crossed to produce embryos carrying mutations in both $RXR\alpha$ and either $RAR\alpha$ or γ genes (see below).

Postnatal Growth Deficiency in $RXR\alpha$ +/- Animals

It was noticed that $RXR\alpha$ heterozygote mice were often smaller than their WT littermates, even though analysis of the weights of 42 animals at birth did not reveal any significant differences between $RXR\alpha$ heterozygote and WT pups (not shown). Animals were thus weighed at 2-3 weeks of age, and the weight of each mouse was expressed relative to the mean weight of WT offsprings in the same litter, which was taken as 1 (Fig. 17). It is readily apparent that the weight distributions of heterozygous and wild type animals are different, heterozygous animals being on average 10% smaller than their WT littermates. In addition, a significant proportion of $RXR\alpha$ +/- mice were severely growth-deficient, with weights of less than 80% that of average WT siblings (see Fig. 17, 0.5 to 0.8 classes; note that such a pronounced growth deficiency was seen at a much lower frequency in WT animals). Several of these runt animals died after a few weeks. The weight deficit observed at 2-3 weeks of age was maintained in surviving adult $RXR\alpha$ +/- animals, which on average also exhibited a 10% weight deficit with respect to WT mice (not shown). Therefore, heterozygosity for the $RXR\alpha$ mutation resulted in postnatal growth deficiency, which was moderate in most animals, but more severe in about 10% of the $RXR\alpha$ +/- mice which were runt and usually die early. A similar growth deficiency phenotype was noticed irrespective of whether the mice were on a pure 129sv or a mixed 129sv - C57/B16 genetic background.

The $RXR\alpha$ Null Mutation is Embryonic Lethal

No living $RXR\alpha$ -/- null mutants could be recovered from litters obtained from $RXR\alpha$ +/- intercrosses (Table V). Furthermore, no living homozygous mutant fetuses were found when delivered by cesarean at 18.5

- 90 -

dpc, suggesting that the RXR α null mutation is embryonic lethal. However, living RXR α ^{-/-} null embryos were obtained at 16.5 dpc and at earlier stages. These embryos were often edemic, slightly smaller than their WT or heterozygous littermates, and had a whiter appearance due to a poor vascular irrigation (compare Fig. 18a with 18b; data not shown). In addition, a significant number of RXR α ^{-/-} animals recovered between 12.5 dpc and 16.5 dpc were dead (in all cases, the death of these embryos was estimated to be posterior to 11 dpc). Embryos (including the dead ones) that could be genotyped at 12.5 dpc and at later stages (which presumably include all the embryos that reached the age of 11 days) showed a significant deficit in the number of RXR α ^{-/-} null embryos (17% instead of the expected 25% frequency). Thus, in addition to the death observed at mid to late gestation, a significant fraction of RXR α ^{-/-} null embryos disappears at earlier stages. However, no deficit of RXR α ^{-/-} embryos could be observed within embryos genotyped at 9.5 and 10.5 dpc, but the fraction of dead or severely retarded embryos at these stages was higher in the RXR α ^{-/-} null mutant population (30%) than in the WT and RXR α ^{+/-} populations (11% and 9%, respectively). These dead RXR α ^{-/-} embryos were often much smaller than their living counterparts and appeared arrested at previous stages of development (not shown). However, given the overall high rate of embryo death at these early stages, it is difficult to distinguish the fraction of dead RXR α ^{-/-} null embryos which died because of their genotype from those that died as part of the 10% population of embryos being "normally" resorbed at these stages. In any case, these data suggest that RXR α performs important functions during early stages of development. The characterization of this "early lethal phenotype" will require careful analysis of a large number of RXR α null embryos at these stages.

Several RXR α null embryos were examined histologically at all stages between 10.5 and 16.5 dpc. Besides cardiac and ocular malformations (see below), the development of several organs appeared delayed with respect to WT or heterozygote embryos. These included the liver and the lungs (after 13.5 dpc) and the skin (at 16.5 dpc), which were all delayed by about 24

hours (data not shown). This developmental delay was also observed for the fusion of the palatal shelves and development of the teeth (not shown).

Table V Viability of RXR α Mutants						
stage (dpc)	RXR α genotype					
	+/+	+/-	-/-	+/+	+/-	-/-
9.5	4	10 (2)	5 (3)	37 (4)	56 (5)	37 (12)
10.5	34 (4)	46 (3)	32 (9)	[28%]	[43%]	[28%]
11.5	21 (3)	47 (2)	12 (5)			
12.5	29	31 (2)	13 (2)			
13.5	11 (2)	36 (3)	15 (6)	132 (3)	247 (11)	79 (35)
14.5	41 (1)	86 (2)	25 (10)	[29%]	[53%]	[17%]
15.5	23	36 (2)	13 (6)			
16.5	28	58 (2)	13 (11)			
18.5	4 (1)	8	1 (1)			
New-born	8	29	0			
The number of dead or resorbed embryo and fetuses is given in parentheses; the distribution of RXR α ^{+/+} , RXR α ^{+/-} and RXR α ^{-/-} animals is given in brackets.						

Heart Malformations in RXR α Null Embryos

Histological analysis of 13.5-16.5 dpc embryos revealed several defects in the heart of RXR α null mutants. It is likely that the mid to late gestation lethality of RXR α ^{-/-} embryos is due to these malformations which result in functionally impaired hearts.

In WT embryos the formation of a trabecular layer of myocytes in the ventricular myocardium begins around day 9.5 of gestation (Challice *et al.*, *Tissue & Cell* 6:447-462 (1973)). A relatively thin compact or subepicardial layer is formed by 10.5 dpc, to further enlarge during the next days. In almost all RXR α ^{-/-} embryos examined between 13.5 and 16.5 dpc, the thickness of the compact layer (CL) of the myocardium was markedly reduced compared with WT embryos, while in comparison the trabecular layer (TL)

- 92 -

appeared more normal (compare CL and TL in Figs. 19a and 19e with Figs. 19b and 19f). This reduction in thickness of the compact layer was variable from embryo to embryo and in the more extreme conditions the wall of the ventricular myocardium was reduced to a single cell layer. The walls of the atrium were also thinner in mutant animals, but the difference was not as pronounced as in the ventricular wall (not shown). No differences were observed between the myocardium of $RXR\alpha^{-/-}$ embryos and their WT littermates at 10.5 dpc (compare CL and TL in Fig. 19c with 19d) and 12.5 dpc (see Table VI; not shown). However one out of 4 of the 11.5 dpc mutants examined displayed an extremely thin ventricular wall (not shown), thus suggesting a variability in the timing of appearance of this abnormality. Whether this variable expressivity of the thin myocardium phenotype reflects the mixed genetic background of these mutants is unknown. In any event, these variations probably account for the relatively large time window at mid-late gestation stages (11.5-14.5 dpc) during which $RXR\alpha^{-/-}$ mutants die (Table V).

In WT embryos, septation of the ventriculoarterial region is completed by 14.5 dpc. In 2 out of 9 $RXR\alpha^{-/-}$ embryos examined between 14.5-16.5 dpc, the membranous portion of the ventricular septum was incomplete (see IVC in Figure 3h) and the upper part of the muscular ventricular septum was also absent (Table VI; data not shown). This malformation may be related to a delay in development, since it was not observed in the 16.5 dpc mutant examined.

TABLE VI

	Abnormalities in RXR α null mutants examined at various developmental stages (10.5-16.5 dcp)									
	10.5 (6)	11.5 (4)	12.5 (4)	13.5 (5)	14.5 (6)	15.5 (2)	16.5 (1)			
Heart Abnormalities										
Thinner myocardium	0/6	1/4	0/4	4/5	6/6	2/2	1/1			
Interventricular communication	NA (a)	NA (a)	NA (a)	NA (a)	1/6	1/2	0/1			
Eye Abnormalities										
Shorter ventral retina	NA (b)	NA (b)	8/8	10/10	12/12	4/4	2/2			
Ventral rotation of the lens	NA (c)	0/8	8/8	10/10	12/12	4/4	2/2			
Thicker cornea	NA (d)	NA (d)	6/8	10/10	12/12	4/4	2/2			
Absence of anterior chamber	NA (e)	NA (e)	NA (e)	NA (e)	NA (e)	4/4	2/2			
Close roots of eyelids	NA (f)	NA (f)	6/8	10/10	12/12	4/4	2/2			
Abnormal sclera	NA (g)	NA (g)	NA (g)	NA (g)	NA (g)	NA (g)	2/2			
Coloboma of the optic nerve	NA (h)	NA (h)	NA (h)	NA (h)	12/12	4/4	2/2			
Persistent retrolenticular membrane	NA (i)	NA (i)	NA (i)	NA (i)	12/12	4/4	2/2			

The number of embryos/fetuses examined at each time is given in parenthesis.

NA: not applicable. (a): interventricular communication is normally present until 13.5 dpc. (b): the dorsal and ventral retina are not distinguishable until 12.5 dpc. (c): the lens is not detached from the surface ectoderm at 10.5 dpc. (d): the cornea develops after 11.5 dpc. (e): an anterior chamber is not always present until 15.5 dpc. (f): the roots of the eyelids are not distinguishable until 12.5 dpc. (g): a well differentiated sclera is not present until 16.5 dpc. (h): the closure of the choroid fissure is not completed until 14.5 dpc. (i): mesenchymal cells are normally present in the vitreous body until 13.5 dpc.

Differentiation and Proliferation of Cardiac Myocytes in RXR α Null Mutants

The deficit in the ventricular cardiac myocytes population could result from an impaired capacity of RXR α ^{-/-} cells to differentiate into cardiac myocytes or from a reduction in the rate of proliferation of normally differentiated cardiac myocytes. To examine a possible role of RXR α in cardiac myocyte differentiation, *in situ* hybridization analyses were performed on 9.5 and 10.5 dpc RXR α null mutant embryos using cardiac α actin, α myosin heavy chain (α MHC) and atrial myosin light chain (MCLIA) anti mRNA probes. At these stages, these genes are strongly expressed in the heart and constitute therefore markers for cardiac myocyte differentiation. Comparable signals were observed in the hearts of both mutant and WT embryos (data not shown). Therefore the absence of RXR α does not prevent the synthesis of muscle mRNA characteristic of cardiac myocytes.

On the basis of ultrastructural and cytochemical properties, the myocytes located in the compact layer appear less differentiated than those of the trabecular layer (Rumyantsev, *Int. Rev. Cytology* 91:187-273 (1977)). Electron microscopy (EM) examination of trabecular cells of WT 14.5 dpc fetuses showed highly organized myofibrils (data not shown), whereas organized myofibrils were extremely scarce in cells at the outer margin of the compact layer. The first mutant myocardium analyzed by EM was severely affected (comparable to the one shown in Fig. 19b). In this specimen, the cells located in the outer region of the myocardium were already highly differentiated, with well organized myofibrils (with visible Z line, Z in Fig. 20d) and prominent sarcomeric reticulum (SR, Fig. 20d). The ultrastructural aspect of these cells was very similar to that of trabecular cells of WT hearts (compare Figs. 20b and 20d compact layer in mutant with 20e trabecular layer of WT). The myocardium of the second 14.5 dpc mutant examined was more mildly affected and a compact layer could clearly be detected histologically (data not shown). EM examination of the cells at the external margin of this "compact layer" showed that these cells were most often similar to cells located at a similar position in the WT myocardium (not shown). However,

organized myofibrils (with a visible Z line) could occasionally be seen in these cells (not shown), which indicates that they may be in a more advanced differentiation state than in the WT myocardium. Together, these observations show that RXR α null mutants are impaired in their capacity to maintain the cells of the presumptive compact layer in a relative undifferentiated state.

The reduction in the size of the compact layer could be due to a decrease in the rate of proliferation of the cells located at the periphery of the myocardium. The percentage of cells in the process of mitosis in that region in both WT and mutants was determined. Surprisingly, the percentage of mitotic cells was only slightly reduced, if at all, in 14.5 dpc mutants compared with WT (2.38% and 1.82% in the severely and mildly affected mutants respectively, and 2.49% in the WT embryo). The rate of DNA synthesis by monitoring bromodeoxyuridine (BrdU) incorporation in WT and RXR α null mutant embryos at 12.5-13.5 dpc was also investigated. No drastic difference in the proportion of BrdU positive nuclei was observed in the myocardium between the WT and mutant embryos (data not shown).

Heart, outflow tract and aortic arch malformations in double RXR α /RAR γ and RXR α /RAR α mutants

A reduced thickness of the ventricular myocardium was also observed at 14.5 and 15.5 dpc in RXR α ^{-/-} embryos carrying additional heterozygous or homozygous mutations in either the RAR α or RAR γ genes, but this reduction was not more severe than that observed in RXR α ^{-/-} embryos (data not shown). Interestingly, one 14.5 dpc RXR α ^{+/-}RAR γ ^{-/-} mutant fetus (out of 6 14.5 and 15.5 dpc fetuses) displayed a thin ventricular myocardium (Table VII), thus suggesting some functional synergy between RXR α and RAR γ during development of the ventricular myocardium (no myocardium defect was seen in RAR γ ^{-/-} fetuses, see Lohnes *et al.*, *Cell* 73: 643-658 (1993)).

In the one RXR α ^{-/-}RAR γ ^{-/-} null mutant embryo examined at 14.5 dpc, the proximal portion of the aorticopulmonary septum was missing, but both the

- 96 -

aorta and pulmonary trunk could be distinguished in the more distal portion of the heart outflow tract, resulting in a partial persistent truncus arteriosus ("partial" PTA; data not shown). A similar partial PTA was found in 1 out of 6 14.5 dpc $RXR\alpha^{-/-}RAR\alpha^{+/-}$. However, the 15.5 dpc $RXR\alpha^{-/-}RAR\gamma^{-/-}$ mutant embryo exhibited a complete agenesis of the aortopulmonary septum ("complete" PTA). A similar "complete" PTA was also found in the single 14.5 dpc $RXR\alpha^{-/-}RAR\alpha^{-/-}$ mutant that was analyzed, indicating a functional synergism between $RXR\alpha$ and $RAR\alpha$ or γ for the formation of the aortopulmonary septum.

Some of the RXR/RAR double mutants also exhibited various abnormalities of aortic arch derivatives (Table VII, and data not shown). In the 14.5 dpc $RXR\alpha^{-/-}RAR\gamma^{-/-}$ fetus, the right pulmonary artery arose from the innominate artery (not shown). In the $RXR\alpha^{-/-}RAR\alpha^{-/-}$ mutant, the ductus arteriosus was missing. In one out of 6 $RXR\alpha^{-/-}RAR\alpha^{+/-}$ embryos, the right subclavian artery arose from the descending aorta and passed dorsally to the esophagus, resulting in a retroesophageal right subclavian artery (not shown; note that this embryo was different from the $RXR\alpha^{-/-}RAR\alpha^{+/-}$ embryo that displayed the "partial" PTA phenotype). Both the PTA defect and aortic arch abnormalities were never found in single $RXR\alpha$ or $RAR\alpha$ or $RAR\gamma$ null mutants and are therefore specific for double mutants.

TABLE VII

Stages (dpc)	Heart, Aortic arch and eye abnormalities in RXR α /RAR γ and RXR α /RAR α double mutants											
	RXR α ^{+/+} RAR γ ^{-/-}		RXR α ^{-/-} RAR γ ^{+/+}		RXR α ^{-/-} RAR γ ^{-/-}		RXR α ^{+/+} RAR α ^{-/-}		RXR α ^{-/-} RAR α ^{+/+}		RXR α ^{-/-} RAR α ^{-/-}	
	14.5	15.5	14.5	12.5	14.5	15.5	14.5	14.5	14.5	14.5	14.5	15.5
Number of fetuses examined	3	3	2	1	1	1	2	6	1	6	2	2
Heart Abnormalities												
Thinner myocardium	1/3	0/3	1/2	0/1	1/1	1/1	0/2	4/6	1/1	6/6	2/2	
Interventricular communication	0/3	0/3	0/2	NA (f)	1/1	1/1	0/2	4/6	1/4	1/6	1/2	
Persistent truncus arteriosus	0/3	0/3	0/2	0/1	1/1 (a)	1/1	0/2	1/6 (a)	1/1	0/6	0/2	
Aortic Arch Abnormalities												
Eye Abnormalities												
Shorter ventral retina	6/6 (b)	6/6 (b)	4/4	2/2	2/2	2/2	0/4	12/12	2/2	12/12	4/4	
Ventral rotation of the lens	6/6	6/6	4/4	2/2	2/2	2/2	0/4	12/12	2/2	12/12	4/4	
Thicker cornea	2/6	2/6	4/4	2/2	2/2	2/2	0/4	12/12	2/2	12/12	4/4	
Absence of anterior chamber	NA (c)	4/6	NA (c)	NA (c)	NA (c)	2/2	NA (c)	NA (c)	NA (c)	NA (c)	4/4	
Closer roots of eyelids	6/6	6/6	4/4	NA (d)	NA (d)	NA (d)	0/4	12/12	2/2	12/12	4/4	
Eversion of the neural retina	0/6	0/6	0/4	0/2	2/2	2/2	0/4	2/12 (e)	2/2	0/12	0/4	
Coloboma of the optic nerve	1/6	0/6	4/4	NA (g)	2/2	2/2	0/4	11/12	2/2	12/12	4/4	
Coloboma of the iris	0/6	0/6	0/4	NA (h)	2/2	2/2	0/4	2/12 (e)	1/2	0/12	0/12	
Retrolenticular membrane	5/6	6/6	4/4	NA (i)	2/2	2/2	0/4	12/12	2/2	12/12	4/4	
Corneal-lenticular stalk	0/3	0/6	0/4	2/2	2/2	2/2	0/4	0/12	0/2	0/12	0/4	

NA: not applicable. (a): "partial" persistent truncus arteriosus (see text). (b): mild reduction. (c): an anterior chamber is not always present until 15.5 dpc. (d): eyelids are not present in the mutants. (e): unilateral in both fetuses. (f): interventricular communication is normally present until 13.5 dpc. (g): the choroid fissure is not closed at 12.5 dpc. (h): the closure of the choroid fissure is not completed until 14.5 dpc. (i): mesenchymal cells are normally present in the vitreous body until 13.5 dpc.

Eye Defects in RXR α Mutant Embryos

Abnormal eyes were observed bilaterally by external inspection in all RXR α null homozygotes collected at stages posterior to 13.5 dpc (compare Fig. 18c with 18d, 21e with 21f, 22a with 22b, and 23d with 23e; data not shown). The size of the palpebral fissure was reduced and pigmented tissue was less visible in the ventral portion of the eye. Two rotations of the eye globe were also apparent in the mutant eyes: one around a dorsoventral axis towards the snout; and one along a rostro-caudal axis towards the ventral side. These rotations were reflected by the lack of coincidence between the margins of the eyelids and the rim of the pupilla in mutant eyes (compare Fig. 22a with 22b; see also the tridimensional reconstruction, Fig. 23d).

In all RXR α ^{-/-} mutant embryos analyzed histologically between 12.5 and 16.5 dpc (14 embryos), the ventral portion of the retina (VR) was bilaterally reduced in size with respect to its dorsal counterpart (DR, compare Fig. 21c and 21e [WT] with 21d and 21f RXR α ^{-/-}, and Fig. 22a with 22b). Note that the ventral and dorsal retina, which are defined with respect to the horizontal plane passing through the papilia (i.e., the optic nerve exit point) have similar sizes in wild type fetuses. The extent of this reduction was however variable from embryo to embryo. In the 4 11.5 dpc embryos analyzed, the ventral edge of the optic cup was located further away from the surface ectoderm than the dorsal edge (compare Fig. 21a with 21b), which suggests that the ventral portion of the optic cup was already shortened at this stage (note that it becomes possible to distinguish the dorsal and ventral retina only at day 12.5 dpc when the axons emanating from the neural retina start to leave the optic cup). The optic cup was apparently normal at 10.5 dpc (6 embryos; not shown). All RXR α mutant eyes analyzed between 12.5 and 16.5 dpc were also characterized by a ventral rotation of the lens (of about 10°; compared dotted line in the lens of Fig. 21c and 22a [WT] with 21d and 22b RXR α ^{-/-}). This rotation is likely to be a consequence of the reduction of the size of the ventral retina.

- 99 -

In the mouse, the development of the cornea begins at 11.5-12.5 dpc with the migration of mesenchymal cells between the surface ectoderm and the lens vesicle (Pei and Rhodin, *Anat. Rec.* 168:108-126 (1970)). All $RXR\alpha$ ^{-/-} eyes examined at 13.5-16.5 dpc had an abnormal cornea which was characterized by a thickening of the corneal stroma (compare C in Fig. 22a and 22i [WT] with C* in Fig. 22b and 22j $RXR\alpha$ ^{-/-}). This feature was visible both in the dorsal and ventral portions of the cornea, but was always more pronounced ventrally. The cells close to the lens were more compacted than the cells beneath the surface ectoderm, which appeared less differentiated, more loosely organized and often in the progress of migration (CS; Fig. 22j, and electron microscopy data not shown). The thickening of the cornea was also clearly seen in 6 out of 8 eyes examined at 12.5 dpc (compare C in Fig. 21c with C* in Fig. 21d).

The space formed between the cornea and the lens by 15 dpc in WT fetuses is called the anterior chamber. In all $RXR\alpha$ null mutants examined at 15.5-16.5 dpc (3 fetuses), the corneal stromal cells were very close to, or in direct contact with the lens, resulting in a marked reduction in the size or an absence of the anterior chamber (compare Fig. 21e with 21f, and data not shown).

At 13.5 dpc, the eyelids develop ventrally and dorsally to the eye as mesenchymal outgrowths covered by ectoderm. They grow towards each other and fuse by 15.5-16.5 dpc (Juriloff and Harris, *Teratology* 40:59-66 (1989)). Histological examination of $RXR\alpha$ ^{-/-} 13.5-15.5 dpc fetuses, showed that the ventral and dorsal eyelids were always located closer to each other, as compared to their WT counterpart at identical developmental stages, resulting in a smaller palpebral fissure (compare Fig. 22a with 22b; see also the tridimensional reconstructions in Fig. 23d and 23e, and the external aspect of the eye in Fig. 18c and 18d). This "closer eyelid" phenotype may not correspond to a defect of the eyelids themselves, which appeared normal in size, but to a closer position of the sites of origin of the eyelids ("roots of the eyelids"; these sites define also the limit of the future conjunctival space). At 12.5 dpc the origins of the eyelids are already identifiable as indentations of

- 100 -

the surface ectoderm (RE; Fig. 21c). These indentations were located in a more anterior position in mutants when compared to WT, which resulted in a shortening of the distance separating these sites (compare RE in Fig. 21c and 21d). Another aspect of this abnormal position of the roots of the eyelids was a hypoplasia of the conjunctival sac in 15.5 and 16.5 dpc mutants, which was always more pronounced on the ventral side (compare VJ in Fig. 21e and 21f; data not shown).

An additional eye abnormality detected in $RXR\alpha^{-/-}$ mutant embryos corresponded to a poor development of the sclera (compare Fig. 22k with 22l). This defect, as well as the defects affecting the cornea, anterior chamber and possibly eyelids, is likely to result in an abnormal behavior of extraocular neural crest derived mesenchymal cells, which contribute to the formation of these structures (see also discussion).

In 11.5 to 13.5 dpc WT embryos, the vitreous body communicates with the periocular mesenchyme through the optic fissure. At this stage mesenchymal and vascular cells which enter through the optic fissure, are present in the vitreous body. At 14.0 dpc, the closure of the optic fissure is completed (Pei and Rhodin, Anat. Rec. 168:108-126 (1970)). In all $RXR\alpha^{-/-}$ mutant eyes examined between 14.5-16.5 dpc (9 fetuses), the closure of the optic fissure was incomplete, leaving a small communication between the vitreous body and periocular mesenchyme, resulting in a coloboma of the optic nerve (CON; compare Fig. 21e and 22a [WT] with 21f and 22b $RXR\alpha^{-/-}$). This cleft allowed mesenchymal cells to enter the vitreous body, resulting in the presence of a large number of mesenchymal cells in this cavity (persistent retrolenticular membrane, F in Fig. 21f and 22b). In WT embryos such cells are normally abundant until 13.5 dpc, to become scarce at 14.5 dpc (Fig. 22a) and completely disappear at 15.5 dpc (Fig. 21e). It could be proposed that both coloboma of the optic nerve and the persistent retrolenticular membrane could result from a general developmental delay in $RXR\alpha^{-/-}$ embryos at the 14.5 dpc stage. However, this is unlikely since these abnormalities were also detected in fetuses at 15.5 dpc (Fig. 21f) and 16.5 dpc (Table VI; data not shown).

Similar Eye Malformations Occur in $RXR\alpha$ -/- and $RXR\alpha$ +/- $RAR\gamma$ -/- Embryos

Histological analysis of $RXR\alpha$ +/- $RAR\gamma$ -/- fetuses at 14.5 dpc and 15.5 dpc revealed similar eye malformations than those detected in $RXR\alpha$ -/- embryos. All $RXR\alpha$ +/- $RAR\gamma$ -/- fetuses were characterized by a closer position of the root of the eyelids (Fig. 22f), which resulted in a reduction of the size of the palpebral fissure. This feature was readily recognizable by external inspection in two fetuses out of 6 (Fig. 18e). The size of the ventral retina was also moderately reduced in all these mutants (compare Fig. 22a [WT] with 22f [mutant]), and the lens displayed a ventral rotation of comparable magnitude as the one seen in $RXR\alpha$ -/- eyes (compare Fig. 22f with 22b). Only one eye out of 12 (Table VII) showed a coloboma of the optic nerve. However, most $RXR\alpha$ +/- $RAR\gamma$ -/- eyes exhibited a fibrous retrolenticular membrane (Table VII). A thickening of the corneal stroma was observed in 4 eyes out of 12 (compare Fig. 22a and 22b with 22f) and the development of the anterior chamber was impaired in 4 out of 6 eyes (Table VII). Therefore $RXR\alpha$ +/- $RAR\gamma$ -/- embryos can exhibit all of the eye malformations seen in $RXR\alpha$ homozygote null mutants, albeit often in a milder form. Note in this respect that $RAR\gamma$ -/- (Lohnes *et al.*, *Cell* 73:643-658 (1993)) and $RXR\alpha$ +/- embryos (not shown) never displayed any eye malformation. These observations suggest the existence of a functional synergy between $RXR\alpha$ and $RAR\gamma$ at some stages of eye development.

Increased Severity of Eye Malformations in $RXR\alpha$ -/ $RAR\gamma$ +/- and $RXR\alpha$ -/ $RAR\gamma$ -/- Double Mutants

In 14.5 dpc $RXR\alpha$ -/ $RAR\gamma$ +/- (2 fetuses) the reduction in size of the palpebral fissure was enhanced with respect to $RXR\alpha$ -/- eyes and the ventral portion of the eye was completely masked by the ventral eyelid (compare Fig. 18d with 18f). Histological examination confirmed that the eyelids were located very close to each other in these embryos compared with the $RXR\alpha$ null mutants (compare VE and DE in Fig. 22c and 22b). The ventral portion

of the cornea was absent and replaced with a thick portion of mesenchymal tissue, whereas the dorsal aspect of the cornea was still identifiable, but markedly reduced in size with respect to $RXR\alpha^{-/-}$ eyes (compare C* in Fig. 22c and 22b). The ventral rotation of the lens was also accentuated in these mutants (compare dotted line in Fig. 22c and 22b).

In the 14.5 and 15.5 dpc $RXR\alpha^{-/-}RAR\gamma^{-/-}$ fetuses, the eyes were entirely buried beneath mesenchymal tissue and thus had a blurry external appearance (Fig. 18g). The cornea and anterior chamber were absent and replaced by a thick layer of mesenchymal tissue which was in direct contact with the lens (Fig. 22d). This internal location of the eye behind a thick layer of mesenchymal tissue was already seen in the eyes of the 12.5 dpc double mutant (Fig. 22e). The eyelids were also not formed in the double mutant fetuses. The invagination of surface ectoderm at the lower and upper part of the eye (which corresponds to the anlage of the conjunctival sac and defines the region of formation of the future eyelids) was absent in both eyes of the $RXR\alpha/RAR\gamma$ double null 12.5 dpc embryo examined (compare Fig. 22e with 21c [WT] and 21d $RXR\alpha^{-/-}$). The ventral rotation of the lens was also markedly enhanced in these mutants and this was correlated with a increased reduction in the size of the ventral retina (see dotted line in Fig. 22d).

Besides these defects which correspond to more severe forms of defects already found in $RXR\alpha$ null mutants, additional malformations were detected in $RXR\alpha^{-/-}RAR\gamma^{-/-}$ double mutant eyes that were never seen in $RXR\alpha^{-/-}$ eyes. Both the 14.5 and 15.5 dpc double mutants displayed a bilateral eversion of the retina (ER; Fig. 22d) at the level of the ventral iris, resulting in an absence of the ventral iris and a coloboma of the iris (Fig. 23f and 23i). All $RXR\alpha^{-/-}RAR\gamma^{-/-}$ eyes also displayed a persistent corneal-lenticular stalk (CL; Figs. 22d, 22e and 23f). This malformation corresponds to a failure of the lens and the surface ectoderm to separate, an event which is normally taking place by 11.5 dpc.

Eye Defects in RXR α /RAR α Double Mutants

RXR α ^{-/-}RAR α ^{+/-} and RXR α ^{-/-}RAR α ^{-/-} double mutants displayed additional defects in the ventral portion of the retina. Two out of six 14.5 dpc RXR α ^{-/-}RAR α ^{+/-} fetuses exhibited an unilateral eversion of the retina (ER; Fig. 22h) extending along the line of closure of the choroid fissure from the optic nerve exit point to the iris, thus corresponding to a unilateral coloboma of the pigmented retina. In both cases the ventral iris was absent, resulting in a ipsilateral coloboma of the iris (not shown) which was readily visible externally (CI; Fig. 18h). The 14.5 dpc RXR α ^{-/-}RAR α ^{-/-} fetus displayed unilaterally an eversion of the pigment retina (from the optic nerve exit point to the iris); the other eye showed a similar eversion in which the iris was not affected, which resulted in a bilateral coloboma of the pigmented retina, and an unilateral coloboma of the iris. These observations, together with the fact that eversion of the retina was never observed in RXR α ^{-/-} mutants, suggest that RXR α and RAR α can synergize to control the normal process of closure of the choroid fissure.

In the single 14.5 dpc RXR α ^{-/-}RAR α ^{-/-} double null mutant studied, both the dorsal and ventral retinas of both eyes were reduced in size with respect to WT and RXR α ^{-/-} eyes (compare Fig. 22g with 22a and 22b). It is interesting to note that, in contrast to what was observed in the RXR α /RAR γ double mutants, the corneal malformations were not enhanced in the RXR α /RAR α double mutant eyes (compare C* in Fig. 22g with C* in Fig. 22b and 22c).

Two RXR α ^{+/-}RAR α ^{-/-} 14.5 dpc fetuses were also analyzed; they did not exhibit any eye abnormality (Table VII; data not shown).

Discussion

The results reported here demonstrate that RXR α is an essential gene in the mouse, since all RXR α null mutants die during embryonic development. Most of the RXR α ^{-/-} embryos die at mid to late gestation stages, most

- 104 -

probably from cardiac failure. A significant proportion (about 30%) of RXR α null mutants disappear however earlier before 10.5 dpc. The reason for this early lethality is currently unknown, but it may reflect an important function of RXR α during early mouse development, which can however be rescued in most instances. These results also demonstrate that RXR α exerts a crucial role in the development of the ventricular myocardium and in eye morphogenesis. Several features of the RXR α mutant phenotype, as well as observations made on compound RXR α /RAR mutants point to a convergence in the signalling pathways that depend on RXR α and RARs.

10 *Role of RXR α in the Development of the Eye*

The development of the eye is a highly complex process which involves multiples interactions between neuroectodermal cells (forming the retina, optic nerve and part of the iris), neural crest derived mesenchymal cells (forming the cornea, choroid, sclera, eyelids, stromal part of the iris and surface ectodermal cells (forming the lens and epithelia of the cornea and eyelids). RXR α null mutants display several eye abnormalities. These include malformations of the anterior segment (thickening of the corneal stroma, absence of anterior chamber, closer roots of eyelids and hypoplasia of the conjunctival sac), abnormal sclera, shortening of the ventral retina, ventral rotation of the lens, coloboma of the optic nerve and persistent retrolenticular membrane. It is likely that the rotation of the lens is a direct consequence of the reduction of the size of the ventral retina, since it is known that the position of the equator of the lens depends on interaction with the neural retina.

25 The malformation of the structures of the anterior segment, as well as the malformation of the sclera, affect structures formed from neural crest-derived extraocular mesenchymal cells. In the case of the defects of the cornea and eyelids, a phenotypic synergy was observed between mutations in the RXR α and RAR γ genes, since the malformations of these structures seen in RXR α null animals were markedly enhanced in their severity in mutants

lacking in addition either one or both alleles of $RAR\gamma$, furthermore, $RXR\alpha$ +/- $RAR\gamma$ /- fetuses reproduced the cornea and eyelid defects of $RXR\alpha$ null mutants. The specific expression of $RAR\gamma$ in periocular mesenchymal cells at 9.5-11.5 dpc suggests that abnormal behavior of these neural crest-derived cells is involved in the genesis of these defects. In this respect, the mesectodermal cells in the $RXR\alpha/RAR\gamma$ double null mutant seem to have been completely deprogrammed since they are unable to form, even in a rudimentary manner, structures such as the cornea and eyelids, and just seem to fill the available space (see Fig. 22d and e). The precise developmental processes which are altered in $RXR\alpha$ null or compound $RXR\alpha/RAR\gamma$ mutants may involve abnormal cell proliferation, abnormal cell migration and/or abnormal cellular interactions.

It is worth stressing that $RAR\gamma$ is not essentially required for the developmental patterning of the anterior portion of the eye since the eyes were correctly developed in $RAR\gamma$ null mutants (Lohnes *et al.*, *Cell* 73:643-658 (1993)). The occurrence of an abnormal corneal stroma, a hypoplastic conjunctival sac and the lack of an anterior chamber in double $RAR\alpha2\gamma$ or $RAR\beta2\gamma$ mutants points to a functional redundancy between $RAR\gamma$ and other RARs in the regulation of the corresponding developmental processes. However, the fact that no malformation of neural crest derived structure occurred in $RAR\alpha\beta2$ double null eyes, as well as the fact that $RXR\alpha/RAR\alpha$ double null eyes did not exhibit more severe malformations of these structures than single $RXR\alpha$ null eyes, argues in favor of $RAR\gamma$ as being the "major" RAR involved in the development of the anterior segment of the eye. Whether $RAR\gamma$ is also involved in the development of the sclera could not be determined from these mutants, but is likely since this structure was not formed in double $RAR\alpha\gamma$ and $\beta2\gamma$ mutants.

The shortening of the ventral retina is another defect present in $RXR\alpha$ null mutants for which mutations in $RXR\alpha$ and in $RAR\gamma$ display a clear phenotypic synergy, since this defect occurred in all $RXR\alpha$ +/- $RAR\gamma$ /- eyes and was increased in severity $RXR\alpha$ /- $RAR\gamma$ +/- and $RXR\alpha$ /- $RAR\gamma$ /- double mutants. The involvement of $RAR\gamma$ (which is not expressed in the retina itself

but in the surrounding extraocular mesenchyme) in the genesis of this defect suggests that this abnormality may result (at least in part) from a defect affecting extraocular mesenchymal cells. In all RXR α null mutants examined at 11.5 and 12.5 dpc, an aberrant presence of mesenchymal cells was observed in front of the ventral part of the optic cup (see Fig. 21b and d). This ventral invasion of mesectodermal tissue was also dramatically accentuated in the 12.5 dpc RXR α /RAR γ double mutant eyes (Fig. 22e). From these observations and from the genetic data implicating extraocular tissue in the genesis of the shortening of the ventral retina, it can be proposed that aberrant outgrowth of mesenchymal cells in front of the ventral part of the optic cup around 11 dpc results in the "reshaping" of the optic cup with a concomitant "backward push" of its ventral margin. Two additional observations support that mechanism: (i) the optic cup appeared normal in mutant embryos at 10.5 dpc, excluding therefore a defect in the primary formation of the ventral part of the optic cup; (ii) no difference in the rate of proliferation was seen between ventral and dorsal neural retinas of WT and RXR α null mutants at 14.5 dpc. This latter observation suggests that the observed shortening of the ventral retina is unlikely to result from a differential growth between ventral and dorsal retinas. Such a differential growth mechanism would also decrease the relative size of the ventral retina with respect to the dorsal retina over time, which was clearly not observed since the relative shortening of the ventral retina (and the resulting rotation of the lens) was similar in 12.5 and 16.5 dpc mutants.

The *Drosophila* RXR homolog, ultraspiracle (usp), has also been implicated in the proper development of the ventral retina in *Drosophila*. This similarity with the RXR α phenotype may reflect a function of RXR conserved between *Drosophila* and mouse in the dorso-ventral patterning of the eye. In this respect, it is interesting to note that the function of usp is required in non retinal cells (Oro *et al.*, *Development* 115:449-462 (1992)), which is a situation reminiscent of the mechanism proposed above for the reduction of the ventral retina in RXR α mutants.

- 107 -

The coloboma of the optic nerve seen in all RXR α null mutants (after 14.5 dpc) is likely to result from a developmental arrest, since the closure of the optic fissure at this level is completed at 14.0 dpc. Additional defects of the closure of the optic fissure, such as coloboma of the iris and coloboma of the pigment retina, were detected in double RXR α /RAR mutants (Table VII). Together, these observations suggest that RXR α and RARs control processes involved in the closure of the optic fissure. In this respect, a more severe complete coloboma of the retina (which is a failure of the optic fissure to close) was often seen in VAD fetuses and in double RAR α γ null mutants (Warkany and Schraffenberger, *Arch. Ophthalm.* 35:150-169 (1946)). The difference between these severe abnormalities and the milder defects seen in RXR α null or compound RXR α /RAR mutants could reflect either an incomplete expressivity of the phenotype in these mutants, or the involvement of RXR α in only a subset of the series of morphogenetic processes involved in the closure of the optic fissure (Geraets, *Am. J. Anat.* 145:411-432 (1976)). The eversions of the neural retina seen in the RXR α /RAR compound mutants is likely to reflect an aberrant differentiation of the outer layer of the optic cup into neural retina. Interestingly, such an aberrant differentiation has also been observed in RAR α γ double mutants as well as in zebrafish embryos treated with RA. These observations suggest a role for RA, RARs and RXR α in the determination of the differentiation state of the neural and pigment retina.

The persistent retrolenticular membrane defect seen in RXR α null mutants could result from the penetration of mesenchymal cells through the cleft resulting from the coloboma of the optic nerve or from a failure of the primary vitreous to degenerate. The occurrence of such a persistent retrolenticular membrane in RXR α \pm -RAR γ \pm mutants in the absence of coloboma of the optic nerve supports that latter possibility.

Function of RXR α in the Development of the Heart

From 13.5 dpc onwards, a reduction in the thickness of the compact layer of the myocardium was seen in most RXR α null mutants and the

- 108 -

myocardium of mutants appeared essentially "trabecular". The trabeculae, as well as the muscular interventricular septum, also appeared thinner. Therefore a reduction in the number of cardiac myocytes occurs in all the myocardial compartments in $\text{RXR}\alpha$ null mutants. Electron microscopy examination of $\text{RXR}\alpha$ -/- mutant and WT myocardial cells at 14.5 dpc revealed two interesting features: (i) a more advanced degree of differentiation of the cells of the "compact" (subepicardial) zone in mutants; (ii) looser compaction between the myocyte cells in the mutant compared to WT, with frequent wide gaps between the cellular membranes of neighboring cells. It is possible that this weaker "cementing" of the cells which would normally contribute to the compact layer could lead them to adopt "trabecular-like" morphological structures and thus account for the trabecular aspect of the myocardium of mutants.

It was observed that cardiac myocytes proliferate at a comparable rate in WT and $\text{RXR}\alpha$ null mutants at 12.5 - 14.5 dpc. This is in apparent contradiction with the markedly reduced cell number in the mutant myocardium after 13.5 dpc. Two possible mechanisms could be considered to explain this paradox. (i) The proliferation of cardiac myocytes may actually be slightly reduced in mutants (for instance by 10-20% which cannot be excluded by the preliminary data). Such a difference could result in a substantial decrease in the cell number in the mutant relative to WT after several generations. This model is supported by the more advanced differentiation state of the cardiac myocytes in mutants since it is known that the rate of proliferation of cardiac myocytes decreases with differentiation. Careful measurements of the growth rates of the ventricular myocytes in several WT and mutants should in the future clarify that possibility. (ii) In the absence of any significant difference in the growth rate between WT and mutant cardiac myocytes, one has to assume that the reduced number of cells in the myocardium of mutants results from loss of cells. In this respect, no evidence was found for cell death in the myocardium of $\text{RXR}\alpha$ null mutants (from histological and EM examination). It is possible also that the looser

cellular contacts observed in mutants could lead to the detachment of some myocardial cells, which may then be carried away in the circulation.

Retinoic acid is a signal implicated in myocardial development since a "spongy myocardium" is among the congenital defects found in the offsprings of VAD dams. Therefore, the myocardial defect observed in RXR α null mutants could reflect the impairment of a retinoid dependent process involved in the correct development of the myocardium. In this respect, a drastic reduction of the compact layer of the myocardium has also been seen in some double RAR α γ mutants. In addition, the occurrence of a thin myocardium in a single 14.5 dpc RXR α +/- RAR γ -/- double mutant fetus suggests that both RXR α and RARs could be involved in the regulation of this RA-dependent process (see below).

Mutations of N-Myc and the Wilms' tumor gene WT-1 have also been shown to produce a hypoplasia of the ventricular myocardium. Whether the pathways affected by these genes and those depending on RXR α are related is currently unknown.

Several RXR α /RAR double mutants were also characterized by aortic arch defects and/or a persistent truncus arteriosus (Table VII). Very similar malformations can be produced in chick embryos by ablation of neural crest cells at the level of rhombomeres 6, 7 and caudal unsegmented portion of the rhombencephalon. Such defects are also found in several types of RAR double mutants and belong to the fetal VAD syndrome (Wilson *et al.*, *Am. J. Anat.* 92:189-217 (1953)). These observations suggest a coordinate role for RXR α and RARs in the RA-dependent processes controlling the normal participation of neural crest derivatives in the formation of the aortico-pulmonary septum and in the determination of the normal pattern of aortic arch derivatives.

Role of RXR α in Retinoid Signaling

The developmental defects seen in RXR α mutants have been described previously in the fetal VAD syndrome. As previously stated, a "spongy

- 110 -

myocardium" was one of the congenital defects resulting from maternal vitamin A deprivation. Shortening of the ventral retina (and ventral rotation of the lens), thickening of the corneal stroma, persistent retrolenticular membrane, closer eyelids and coloboma of the optic nerve are among the most common eye malformations found in offsprings of VAD dams (Warkany and Schraffenberger, *Arcl. Ophth.* 35:150-169 (1946)). These similarities between the defects occurring in RXR α null mutants and those occurring in the VAD state suggest that RXR α is essential for several aspects of vitamin A action. It is also possible that the growth deficiency observed in heterozygote animals corresponds to an impairment of the growth promoting function of vitamin A. RXR α could be implicated in the mediation of the retinoid signal in two ways: either as a receptor for 9cis RA or as a heterodimeric partner required by RARs for efficient binding to retinoic acid response elements. The results presented here do not allow any conclusion to be drawn concerning the possible role of a 9cis RA dependent regulatory function of RXR α in the events perturbed by the RXR α mutation. Two sets of observations (detailed below) support however the idea that RXR α /RAR heterodimers could be implicated in several aspects of RXR α function.

Several defects seen in RXR α mutants fetuses have also been found in double RAR mutants: a trabecular ventricular myocardium in RAR $\alpha\gamma$ double null fetuses, absence of anterior chamber, hypoplasia of the conjunctival sac, abnormal corneal stroma, absence of sclera and coloboma of the optic nerve in RAR $\beta2\gamma$ and $\alpha\gamma$ double mutants; retrolenticular membrane in most RAR double mutants.

The analysis of compound RXR α /RAR mutants also demonstrates that a genetic interaction between RXR α and RARs is involved in several developmental processes: (i) the same eye and myocardium malformations arise in RXR α α α and RXR α α α /RAR γ γ γ fetuses; (ii) several ocular malformations arising in RXR α mutants are markedly enhanced in their severity when the RXR α null mutation is associated with heterozygous or homozygous null alleles of the RAR γ gene; (iii) malformations not present in single RXR α mutants, such as persistent truncus arteriosus, aortic arch

- 111 -

malformations, eversion of the neural retina, coloboma of the iris or pigment retina or persistent corneal-lenticular stalk, arise in double mutants. The phenotypic similarity between the RXR α mutants and the double RAR mutants, together with the functional synergy between the RXR α and RAR mutations clearly shows a convergence of the functions of RXR α and RARs which supports the concept of RXR/RAR heterodimers as being the entities regulating the corresponding regulatory processes. However, it cannot be excluded that RXR α and RARs regulate distinct processes which could be coordinately involved in the development of the affected structures.

It is worth stressing that several of the above mentioned functional interactions between RXR α and RARs require only heterozygosity for one of the 2 genes. If one supposes that this synergy does not reflect the formation of heterodimers between the two types of molecules, but rather that RXR α and RARs regulate separate events which coordinately control the relevant processes (for instance if RXR α and RARs control respectively the production of a ligand and its receptor), then one would also have to assume that these regulatory functions of RXR α or RARs would be very sensitive to gene dosage. Eventhough such a model cannot be excluded, the data disclosed here fit better with the heterodimer model, in which heterozygosity for one gene reduces the quantity of corresponding heterodimers by half, and thus could bring this quantity below a critical threshold.

The shortening of the ventral retina, ventral rotation of the lens and increased thickening of the ventral portion of the cornea are features consistently present in RXR α null mutants, and which are also part of the VAD syndrome. However, these malformations have never been seen in any of the RAR double mutants described here, including the double RAR α mutants which recapitulate all the other ocular defects of the VAD syndrome. The result from the double RXR α /RAR γ mutants however clearly indicate that RARs have a function in the control of the normal processes affected in this defect (see above). It is therefore possible that there is a extreme functional redundancy among RARs in this system. Alternatively, the crucial role of RXR α in the patterning of the ventral part of the eye could reflect a function

of RXR α not solely involving RXR/RAR heterodimers. This possibility is also in agreement with the complete penetrance of this defect in RXR α +/- RAR γ -/- fetuses, which is difficult to reconcile with the idea of the requirement of only a low level of RXR/RAR heterodimers. In this respect, it has been shown that the embryonic mouse retina synthesizes RA and that the RA-synthesizing enzymes are different in the dorsal and ventral parts of the retina (McCaffrey *et al.*, *Develop. Biol.* 158:390-399 (1993)). It is tempting to speculate that the ventral enzyme could be involved in the synthesis of 9cis RA which could act specifically as a ligand for RXR α .

Specificity, Redundancy and Variation in Expressivity

RXR α has clearly a specific role for the correct development of the ventricular myocardium and several structures of the eyes. In situ hybridisation analysis of the distribution of RXR transcripts during the stages studied here have revealed a uniform low level of expression of both RXR α and RXR β . However, given that the signals detected in this study were close to background hybridization, it was not possible to determine whether RXR α and β transcripts are indeed coexpressed in all tissues. In addition, no information concerning the distribution of RXR proteins is available to date. It is therefore not possible at present to conclude whether the defects seen in RXR α null mutants represent the absence of a specific function of RXR α , that other RXRs cannot fulfill, or represent the reduction below a critical level of RXR (any type) in the affected cells.

The requirement by multiple members of the nuclear receptor family for heterodimerization with RXR for efficient DNA binding has led to the suggestion that RXRs may possess pleiotropic functions involved in the biological action of several ligands (see introduction). The relatively limited phenotype of RXR α null mutants is thus rather surprising. In addition, the defects observed are all related to the developmental function of vitamin A (see above) and do not hint to interference with other signalling pathways. This could suggest the existence of a large degree of functional redundancy

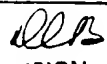
- 113 -

among RXRs for their putative function as heterodimeric DNA binding partners. In this respect, it is noteworthy that almost none of the vast array of defects associated with RAR deficiency are found in RXR α mutants. A functional redundancy among RXRs is also suggested by the fact that some RXR β null mutants are viable and display no apparent abnormality. The occurrence of specific malformations only in the double RXR α /RAR mutants also shows that in the presence of a full set of RARs, other RXRs are able to substitute for the loss of RXR α .

Some variation in the expressivity of the myocardium phenotype (see result section) was observed. Moreover, two RXR α /-RAR α +/ or one RXR α /-RAR γ +/ animals had a myocardium of normal aspect at 14.5 dpc (Table 3). These variations could reflect the mixed 129sv/C57Bl6 genetic background of most of the mutants analyzed. In this respect, it should be noted the compound RXR α /RAR mutants had a larger C57Bl6 contribution than the single RXR α null mutants which in all cases had a 129sv contribution greater than 75%. It will be interesting in the future to see whether changing the genetic background of RXR α mutants will alter the expressivity and penetrance of the myocardial defect.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>29</u> , line <u>18</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution American Type Culture Collection	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Date of deposit May 6, 1994	Accession Number CRL 11632
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable) The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
<input checked="" type="checkbox"/> For receiving Office use only This sheet was received with the international application	<input type="checkbox"/> For International Bureau use only This sheet was received by the International Bureau on:
Authorized officer DORIS L. BRIDGES  INTERNATIONAL DIVISION	Authorized officer:

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>29</u> , line <u>18</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution American Type Culture Collection	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Date of deposit May 6, 1994	Accession Number CRL 11632
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
Pluripotent Embryonic Cells, ES-D ₃ In respect of those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC).	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
For receiving Office use only	For International Bureau use only
<input checked="" type="checkbox"/> This sheet was received with the international application	<input type="checkbox"/> This sheet was received by the International Bureau on:
Authorized officer DORIS L. BROOK <i>DLB</i> INTERNATIONAL DIVISION	Authorized officer

What is claimed is:

1. A transgenic mouse which has been genetically altered such that said mouse is deficient in the normal expression of one or more members of the RAR or RXR family of receptors.

2. The mouse of claim 1 wherein said mouse does not express detectable levels of a receptor selected from the group consisting of all isoforms of RAR α , all isoforms of RAR γ , RAR α 1, RAR γ 2, RAR β 2 and all isoforms of RXR α .

3. The mouse of claim 1 wherein said mouse does not express a functional receptor selected from the group consisting of all isoforms of RAR α , all isoforms of RAR γ , RAR α 1, RAR γ 2, RAR β 2, and all isoforms of RXR α .

4. A mouse not showing the deficiency as defined in any one of claims 1-3, but wherein said mouse is heterozygous or chimeric for said deficiency.

5. A transgenic mouse which has been genetically altered such that said mouse is heterozygous or homozygous for a deficiency in the normal expression of RXR α and RAR γ receptors or deficient in the expression of RXR α and RAR α receptors.

6. A cell line which is heterozygous or homozygous for a deficiency in the normal expression of one or more members of the RAR or RXR family of receptors.

7. The cell line of claim 6 wherein said cell line does not express detectable levels of a receptor selected from the group consisting of all

isoforms of RAR α , all isoforms of RAR γ , RAR α 1, RAR γ 2, RAR β 2, and all isoforms of RXR α .

8. The cell line of claim 6 wherein said cell line does not express a functional receptor selected from the group consisting of all isoforms of RAR α , all isoforms of RAR γ , RAR α 1, RAR γ 2, RAR β 2, and all isoforms of RXR α .

9. The cell line of claim 6 wherein said cell line is a pluripotent cell line.

10. The cell line of claim 6 wherein said cell line is a totipotent cell line.

11. The cell line of claim 6 wherein said cell line is a pluripotent cell line having the ATCC designation CRL11632.

12. A cell line which is heterozygous or homozygous for a deficiency in the normal expression of RXR α and RAR γ receptors or deficient in the expression of RXR α and RAR α receptors.

13. The cell line of claim 12 wherein said cell line is a pluripotent cell line having the ATCC designation CRL 11632.

14. A method of identifying the subtype or isoform of RAR or RXR receptor bound by an agent, said method comprising the steps of:

- (a) incubating said agent with one or more of the cell lines of claim 6; and
- (b) determining the amount of agent bound by said cell line(s).

15. A method of identifying the subtype or isoform of RAR or RXR receptor bound by an agent, said method comprising the steps of:

(a) incubating said agent with one or more of the transgenic mice of claim 1; and

(b) determining the amount of agent bound by said transgenic mice.

16. A method of identifying an agent which is an antagonist or agonist of a subtype or specific isotype of RAR or RXR receptor, comprising the steps of:

(a) incubating said agent with one or more of the transgenic mice of claim 1; and

(b) determining the effect said agent has on the development of said transgenic mice.

17. A method of identifying an agent which is an antagonist or agonist of a subtype or specific isotype of RAR or RXR receptor, comprising the steps of:

(a) incubating said agent with one or more of the transgenic mice of claim 1; and

(b) determining the effect said agent has on the expression of a RA induced gene sequence.

18. The method of claim 15 wherein said mice have further been altered to contain a DNA sequence comprising a RARE or RXRE operably linked to a marker sequence.

1/48

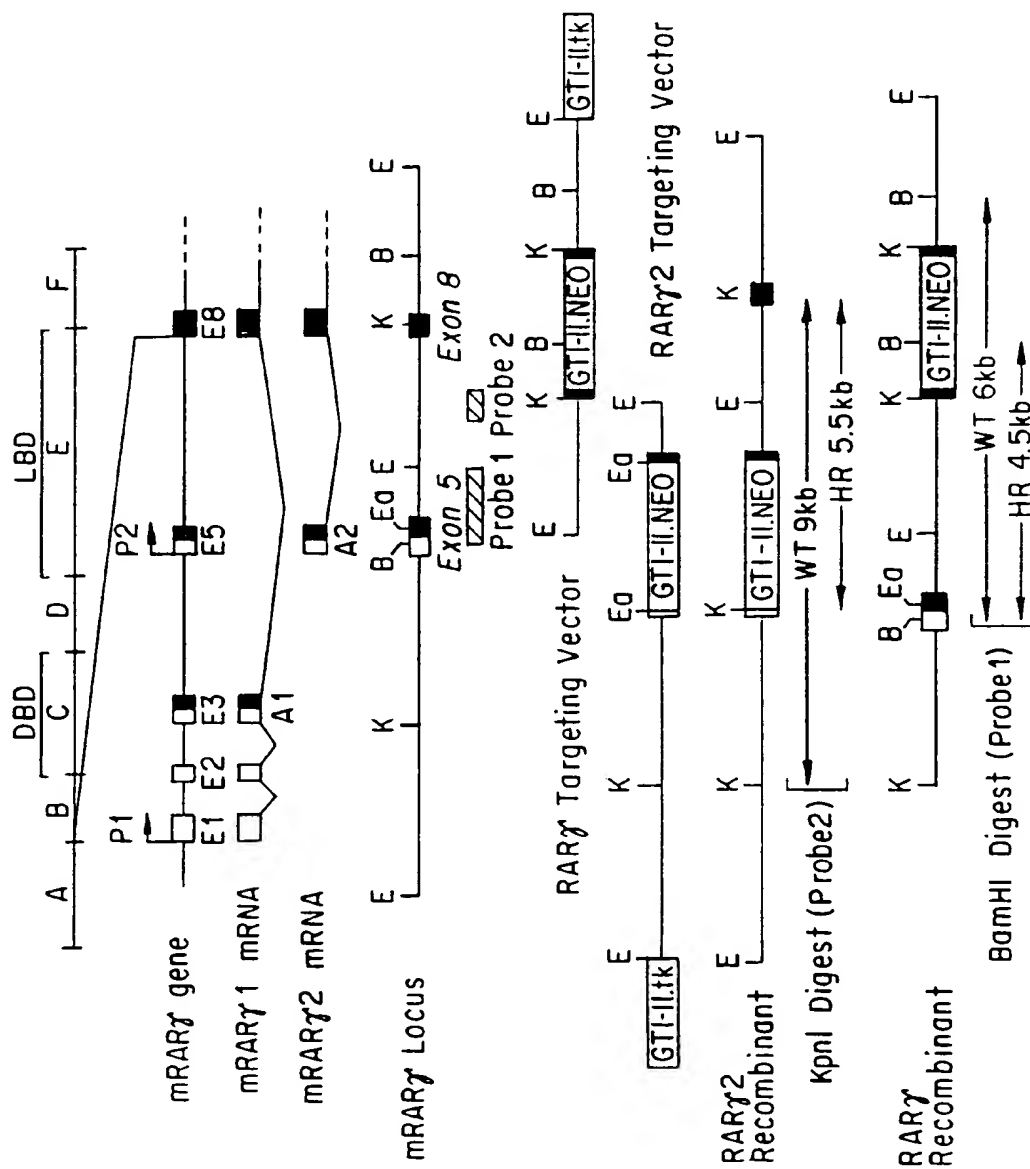
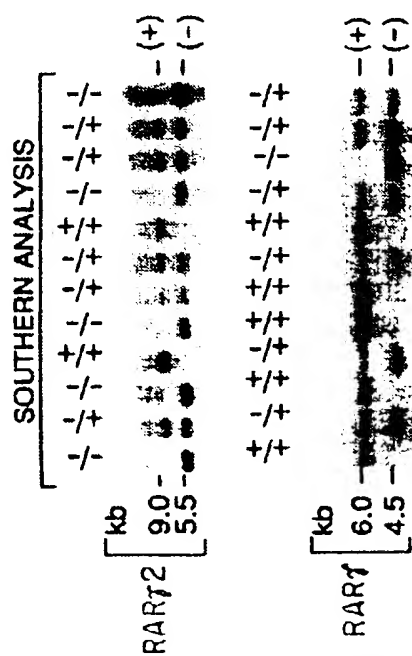
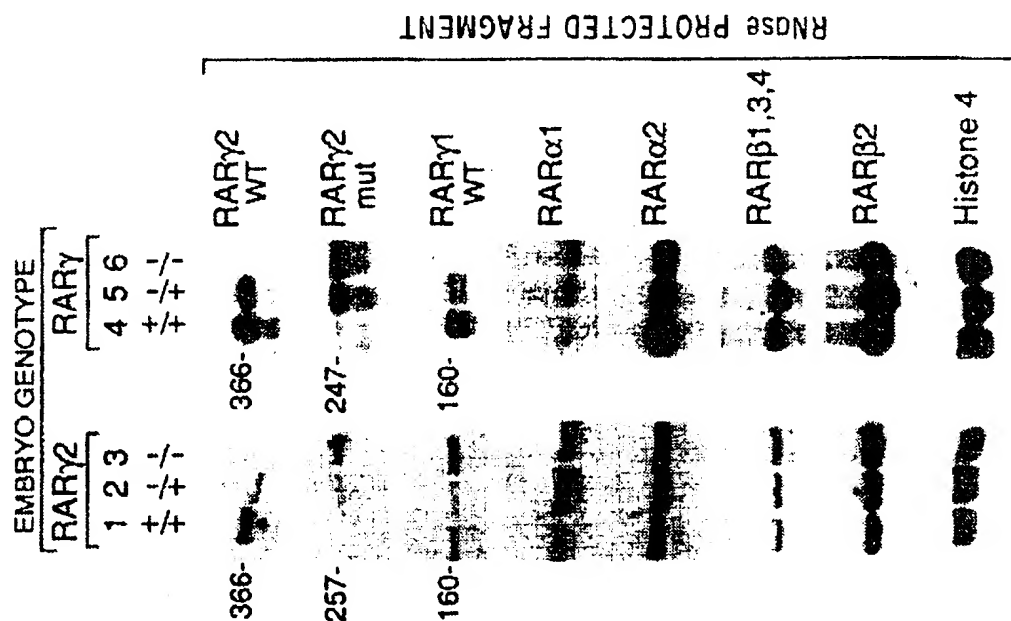


FIGURE 1A



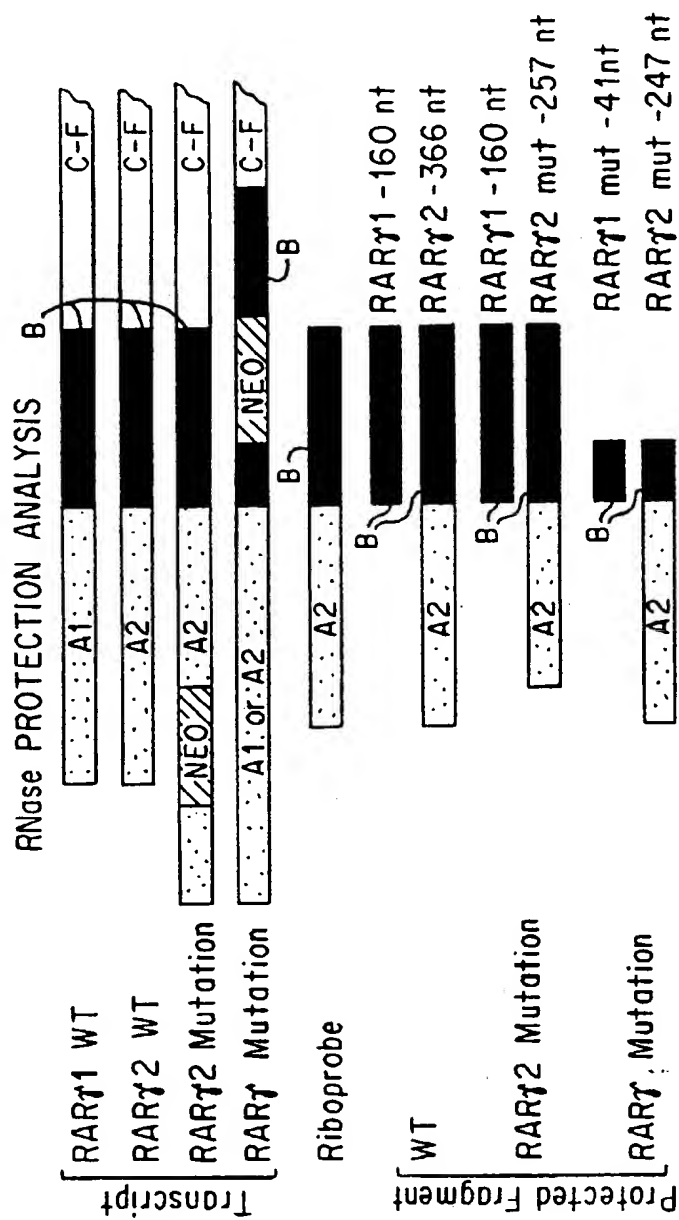


FIGURE 1C

4/48

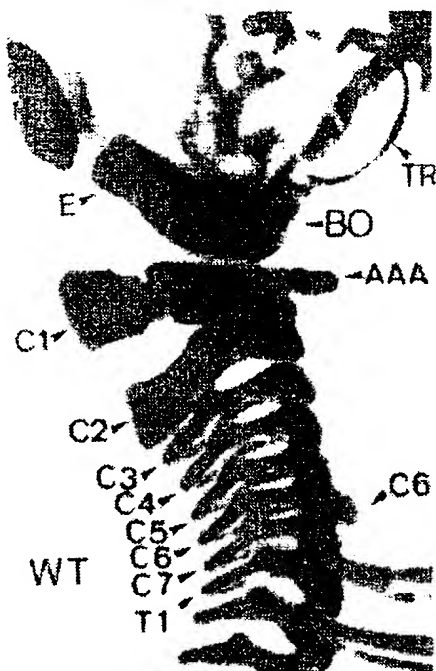


FIGURE 2A

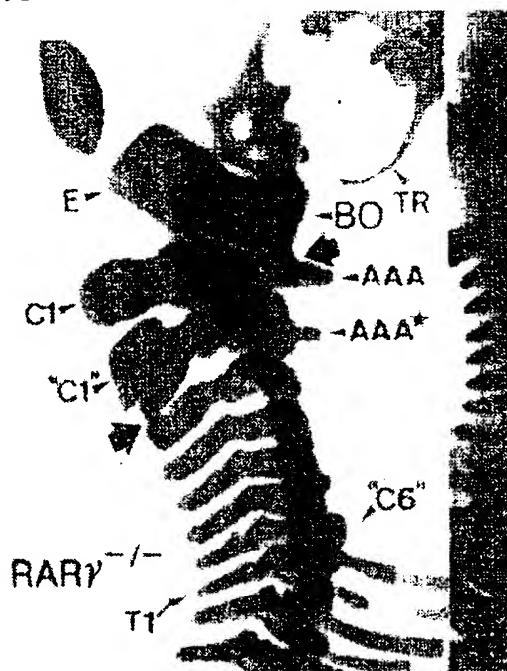


FIGURE 2B

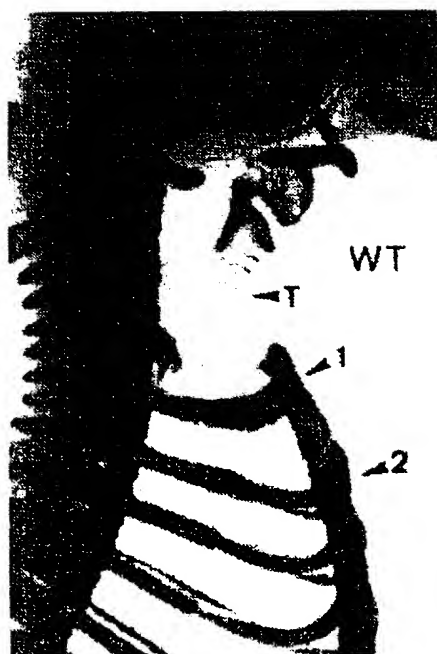


FIGURE 2C



FIGURE 2D

5/48

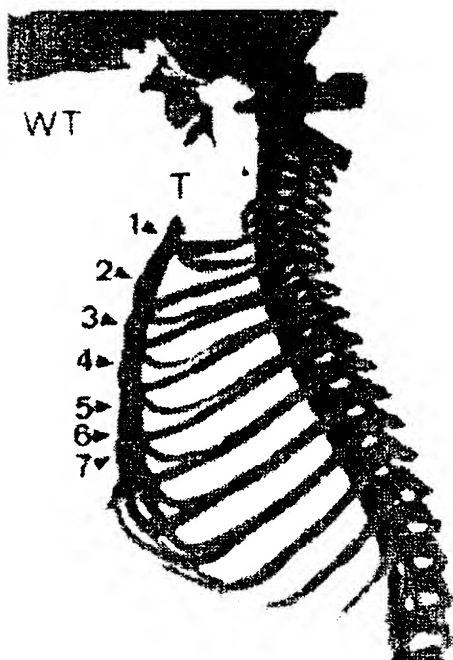


FIGURE 2E

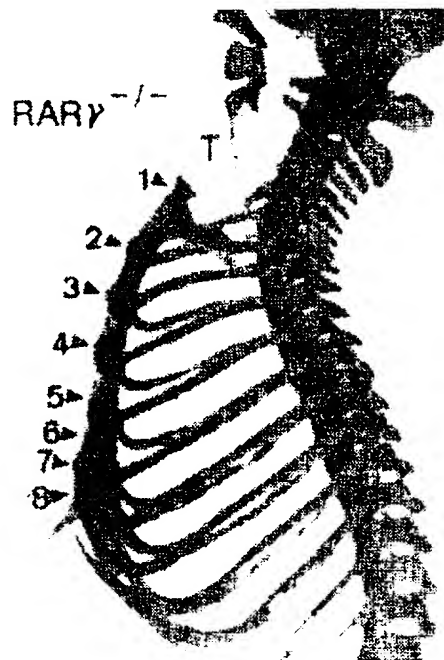


FIGURE 2F

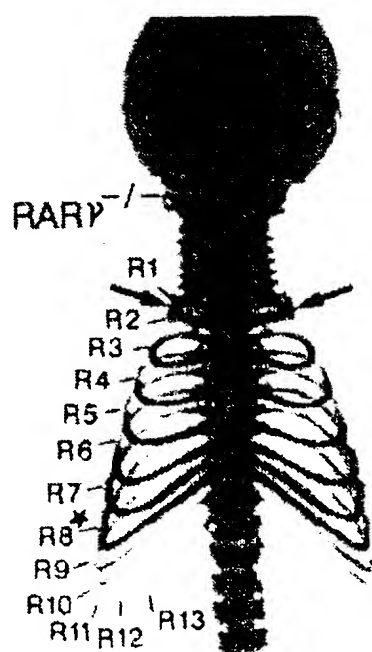


FIGURE 2G

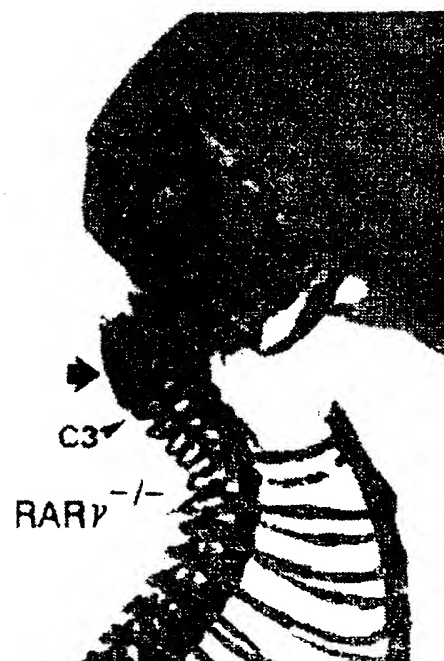


FIGURE 2H

6/48

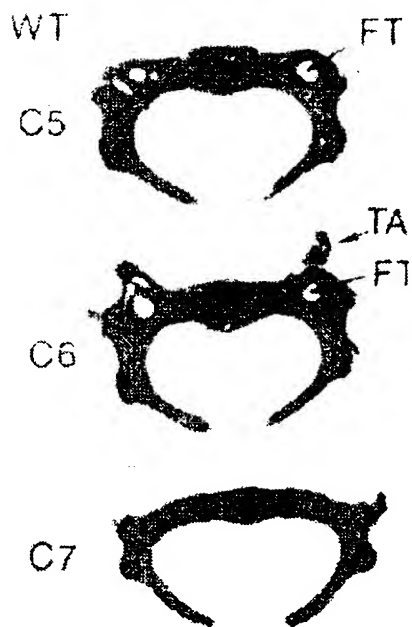


FIGURE 2I

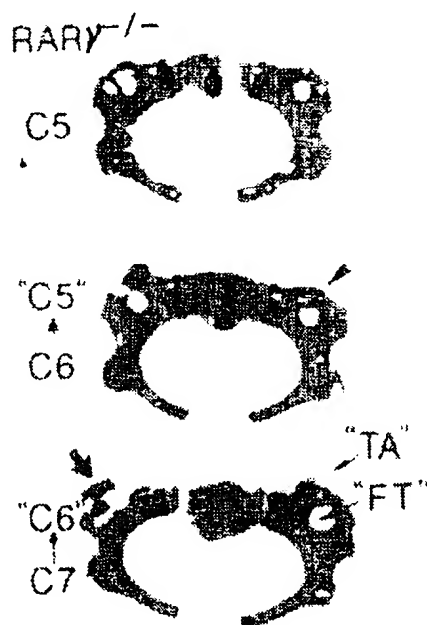


FIGURE 2J

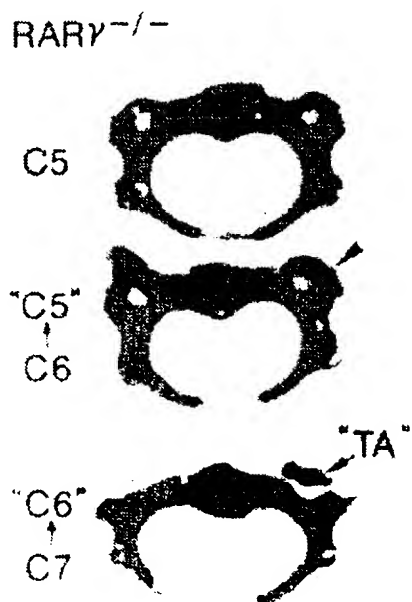


FIGURE 2K

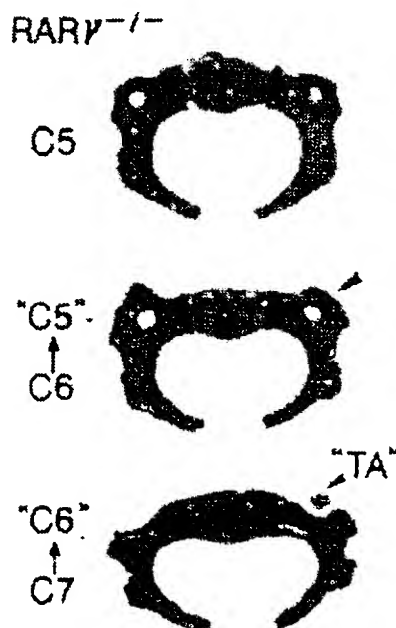


FIGURE 2L

FIGURE 3A



FIGURE 3B

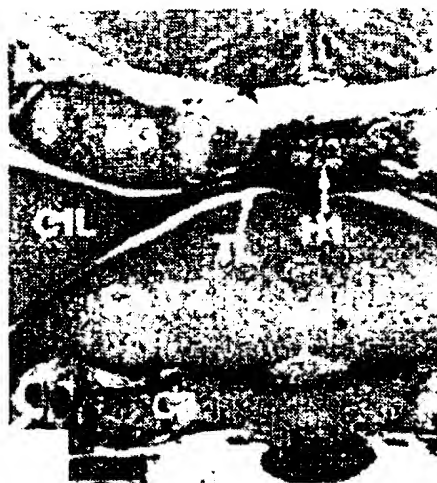


FIGURE 3C

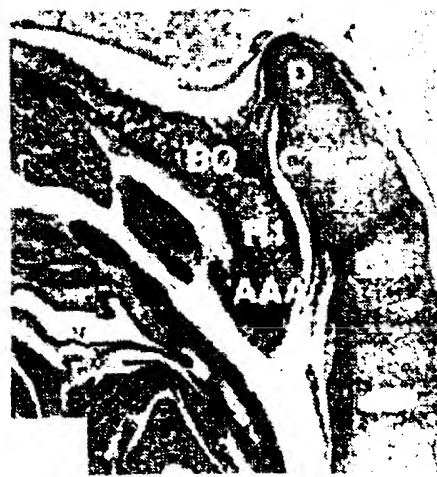


FIGURE 3D



FIGURE 3F

FIGURE 3E

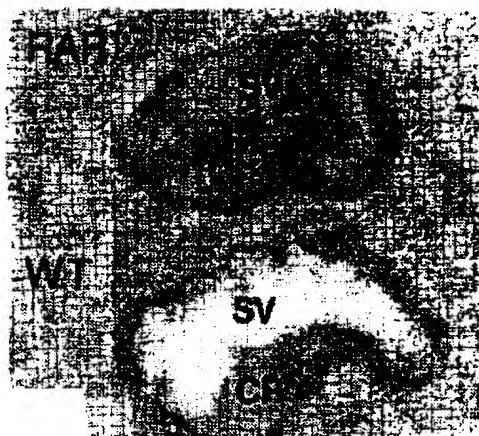


FIGURE 4A



FIGURE 4B



FIGURE 4C



FIGURE 4D



FIGURE 4E



FIGURE 4F



FIGURE 4G

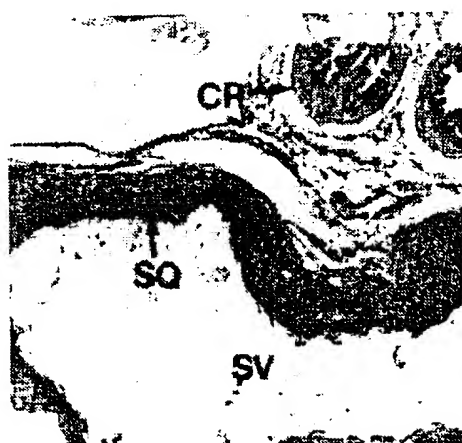


FIGURE 4H



FIGURE 4I

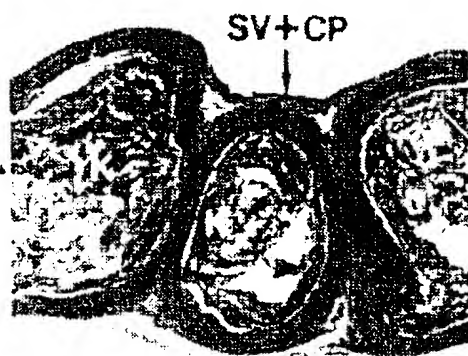


FIGURE 4J



FIGURE 4K



FIGURE 4L



FIGURE 4M

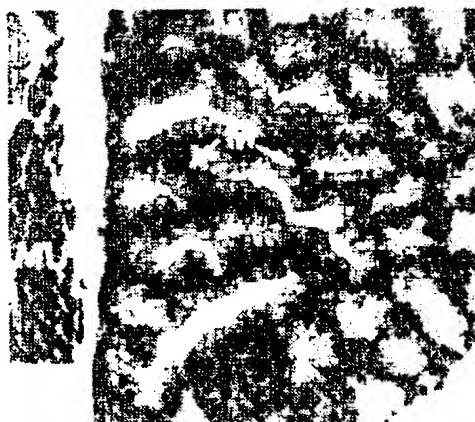


FIGURE 4N



FIGURE 4O

14/48

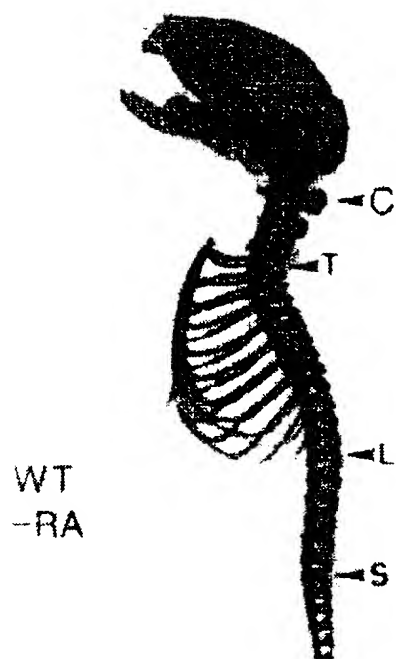


FIGURE 5A

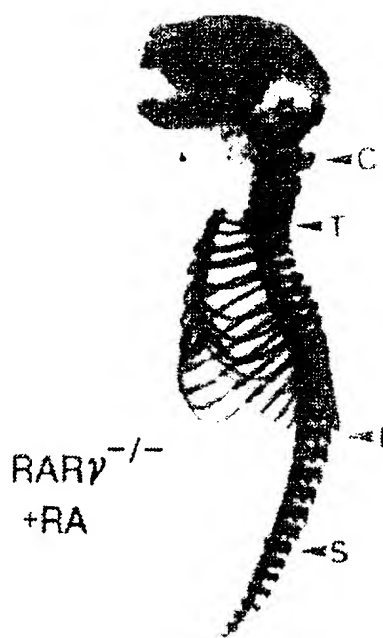


FIGURE 5B

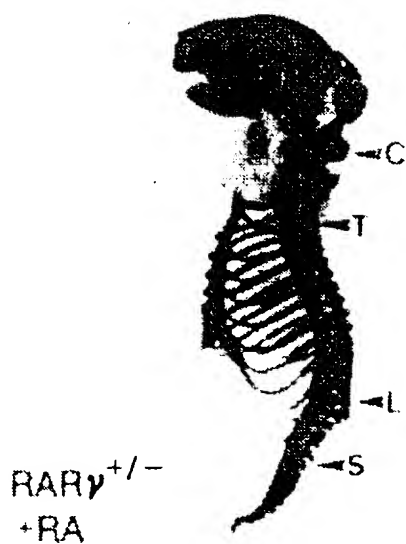


FIGURE 5C



FIGURE 5D

15/48

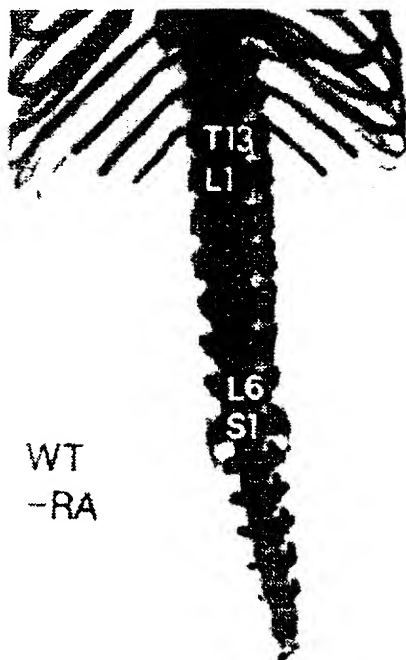


FIGURE 5E

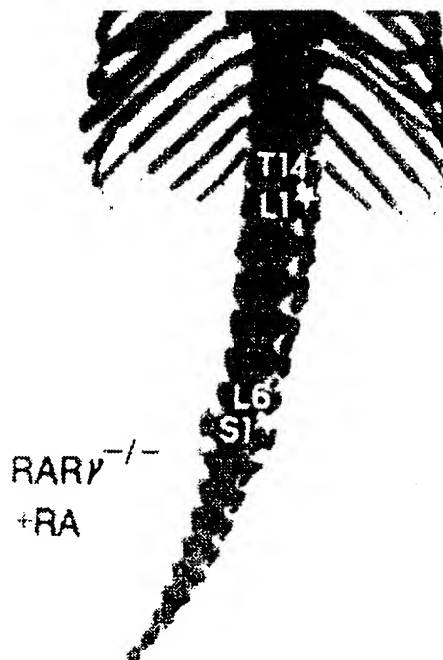


FIGURE 5F

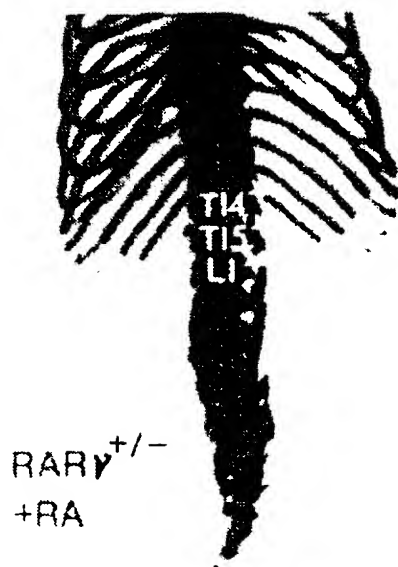


FIGURE 5G

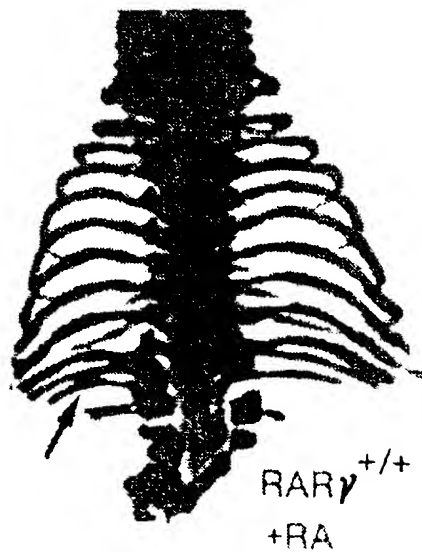


FIGURE 5H

16/48

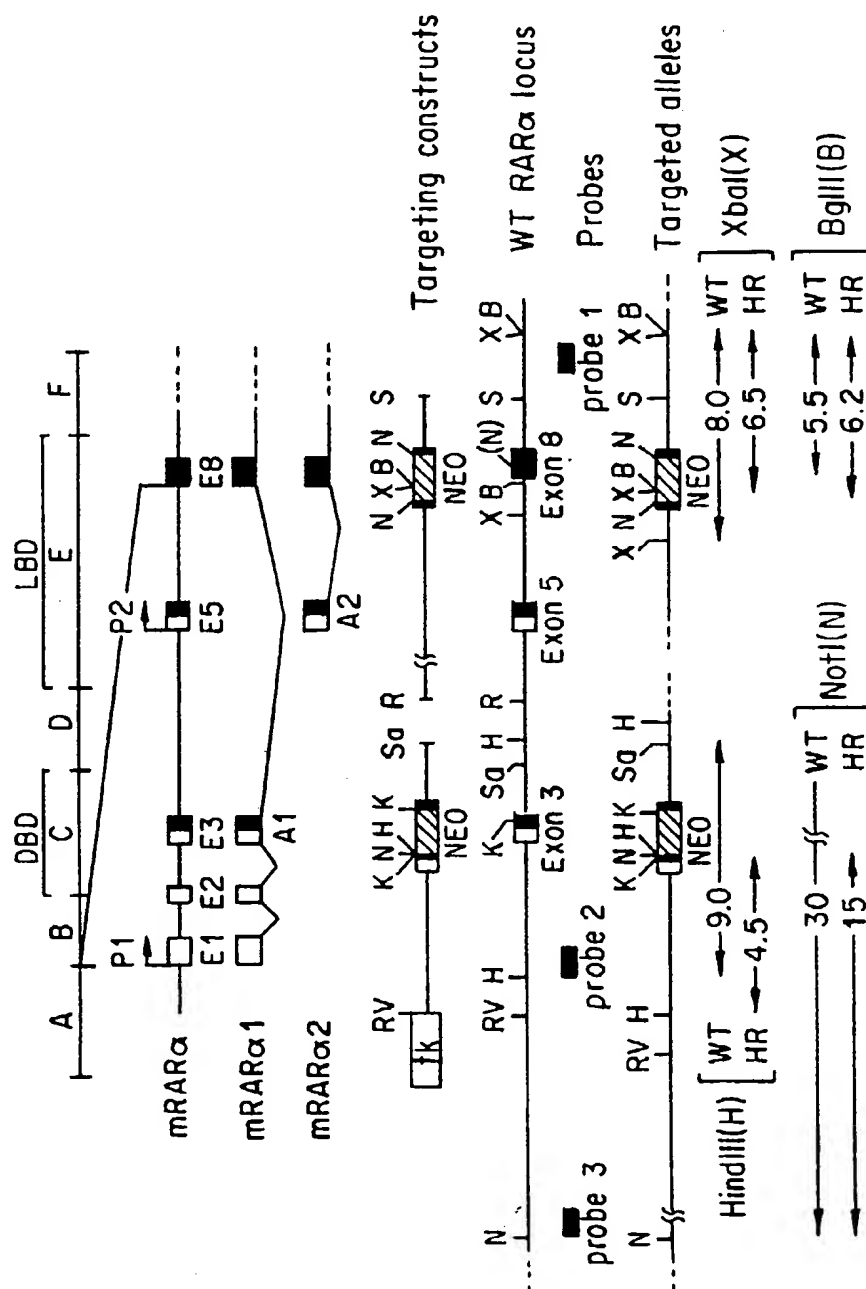


FIGURE 6A

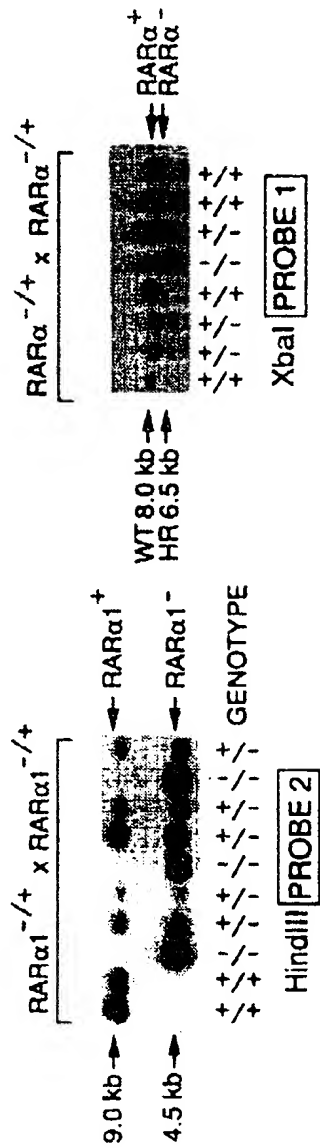


FIGURE 6B

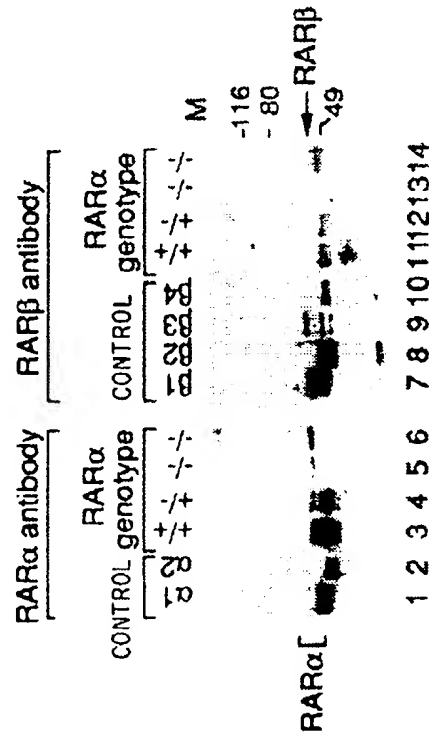


FIGURE 6D

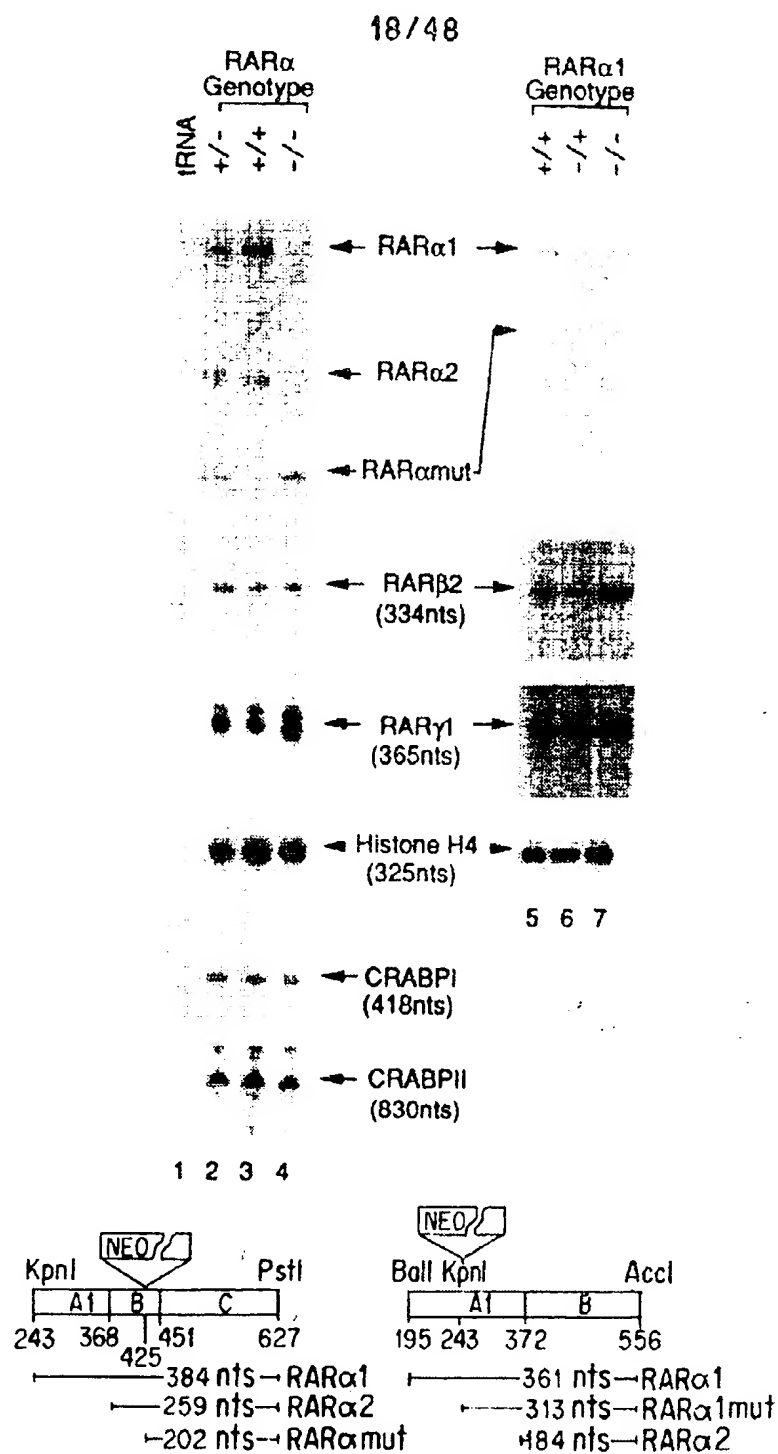


FIGURE 6C

19/48

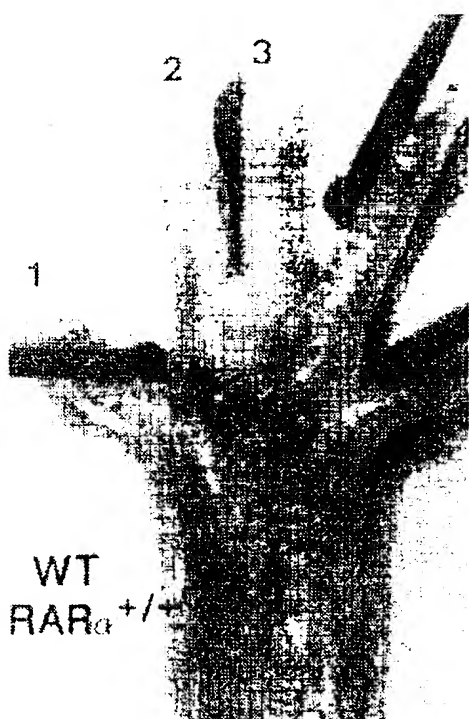


FIGURE 7A

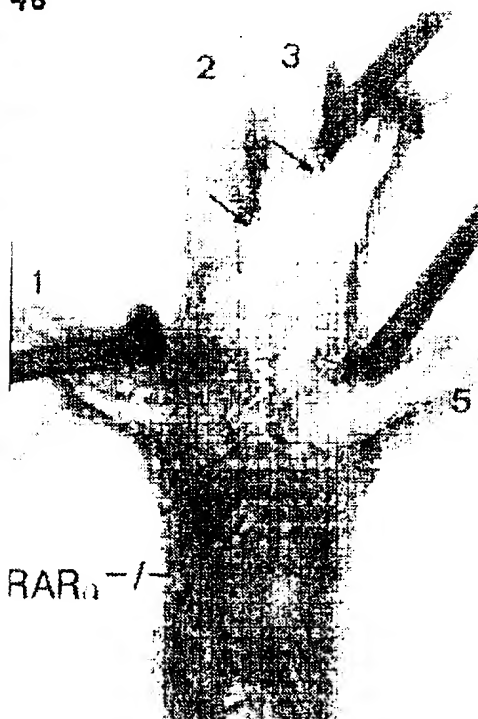


FIGURE 7B



FIGURE 7C



FIGURE 7D

20/48



FIGURE 8C



FIGURE 8B



FIGURE 8A

21/48



FIGURE 8D

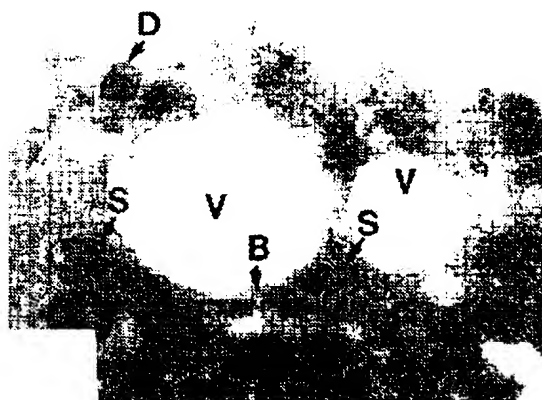


FIGURE 8E



FIGURE 8F

22/48

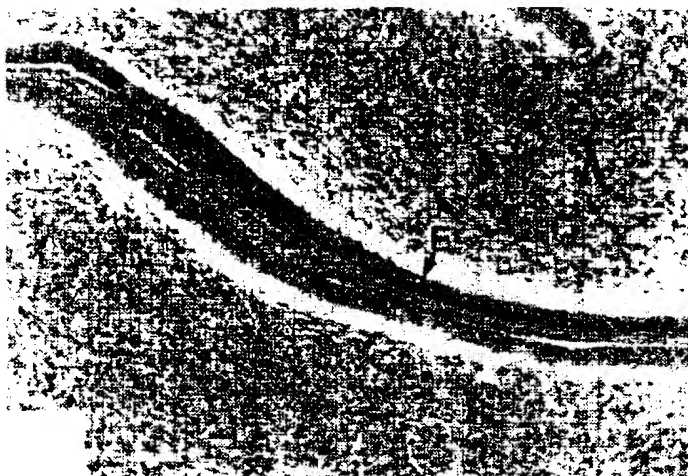


FIGURE 8G



FIGURE 8H

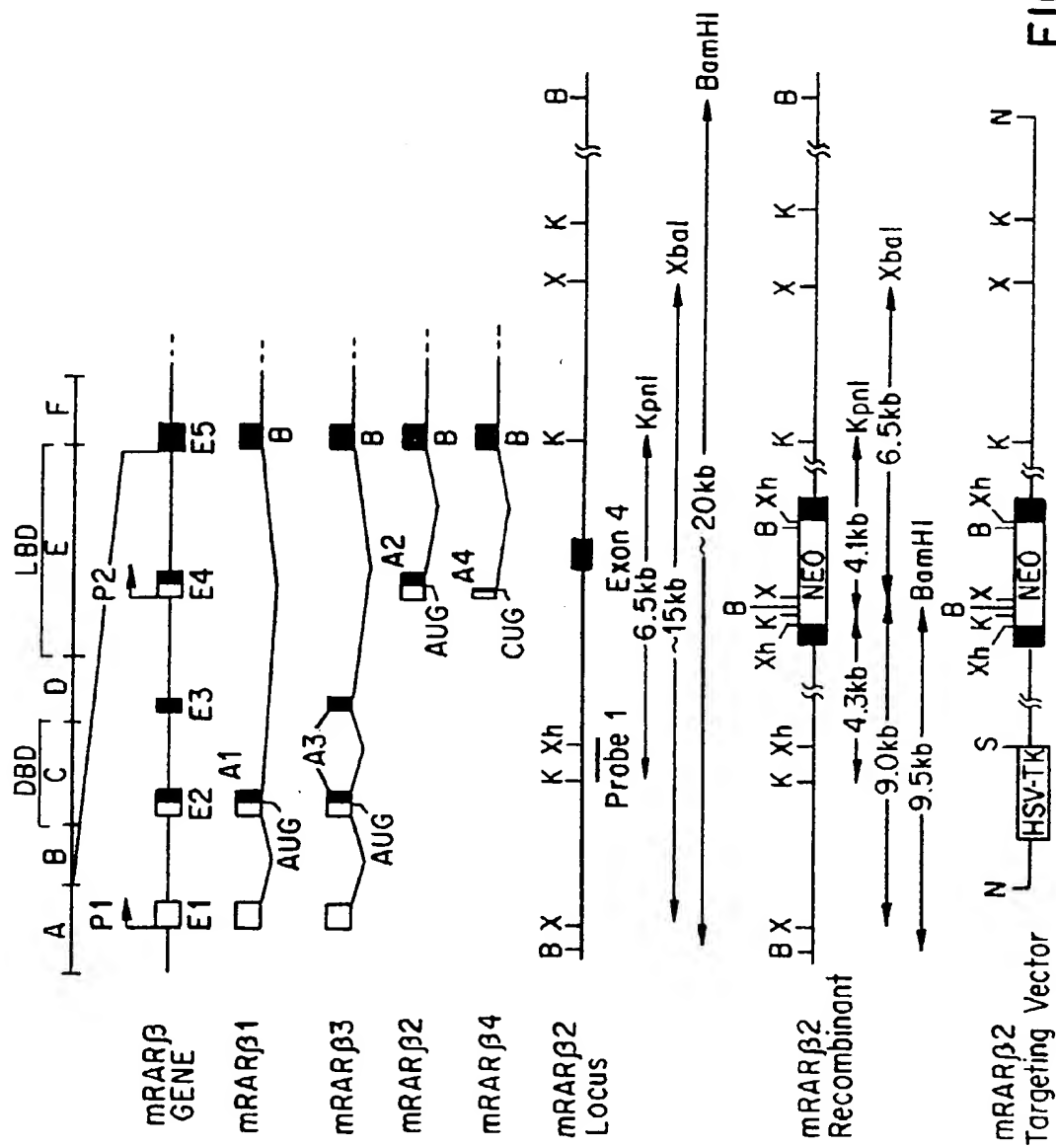


FIGURE 9A

24/48

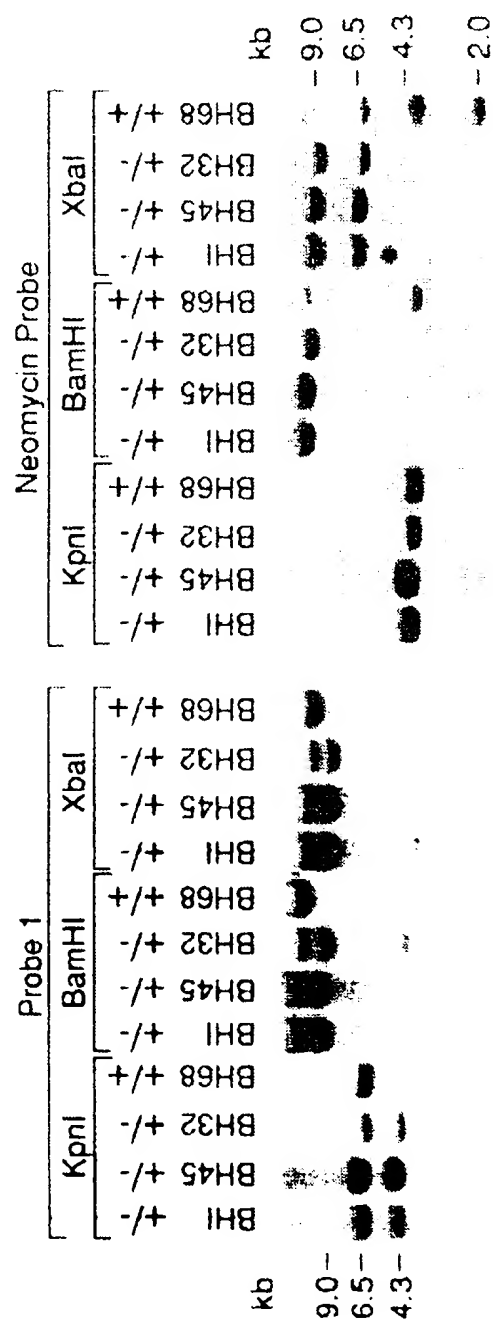
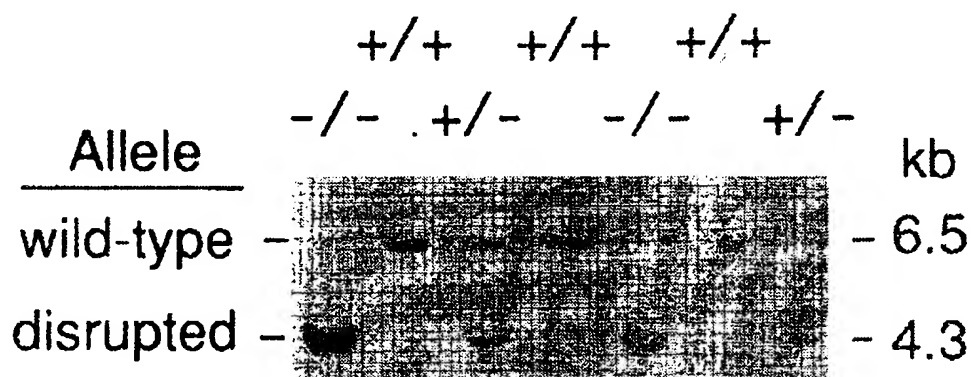


FIGURE 9B

25/48

Genotype of one week-old offspring of
mRAR β 2 heterozygote (+/-) intercrosses



Genotype of one month-old offspring
of mRAR β 2 +/- intercrosses

<u>+/+</u>	<u>+/-</u>	<u>-/-</u>
61(25%)	117(48%)	65(27%)

FIGURE 10

26/48

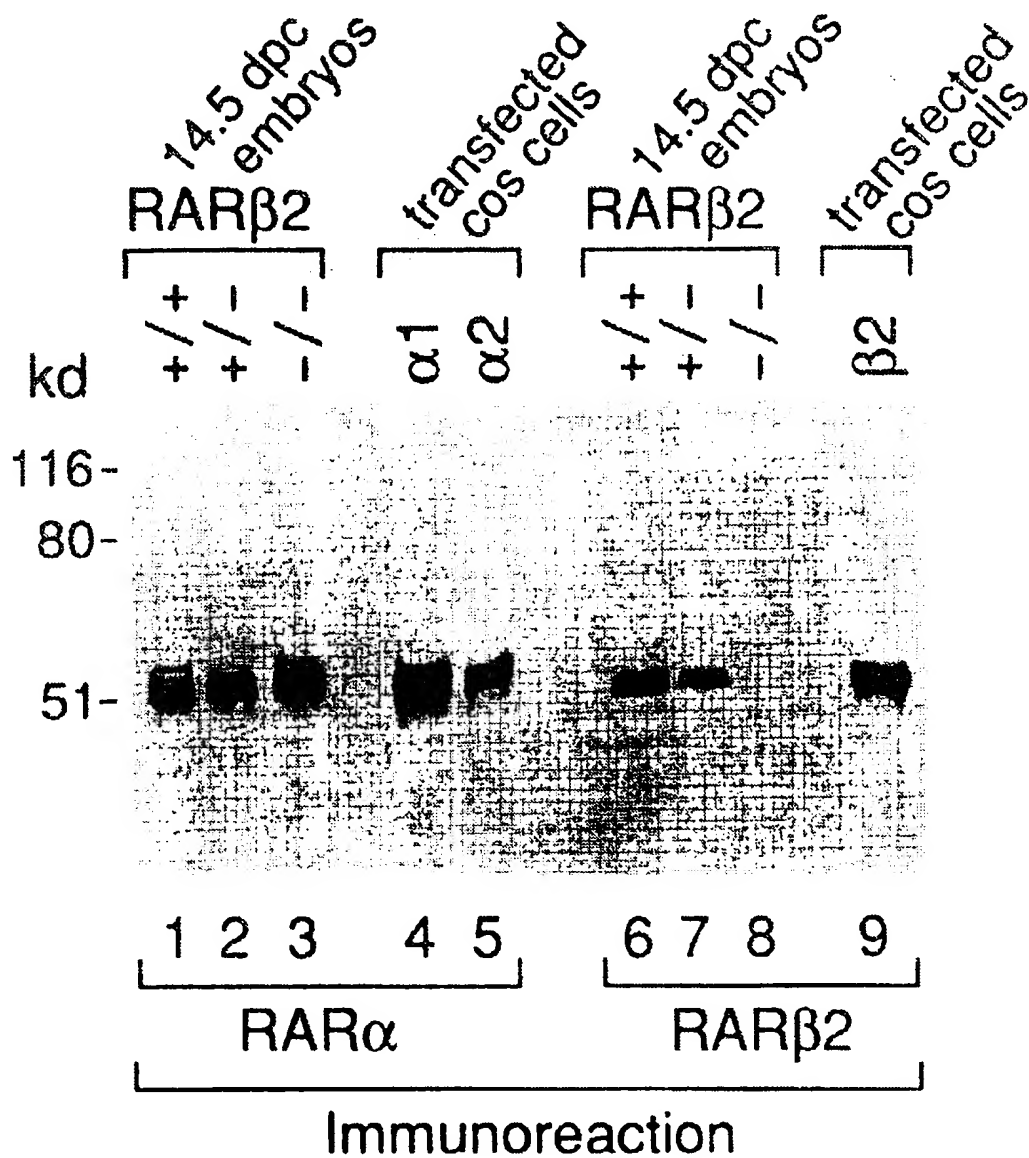


FIGURE 11

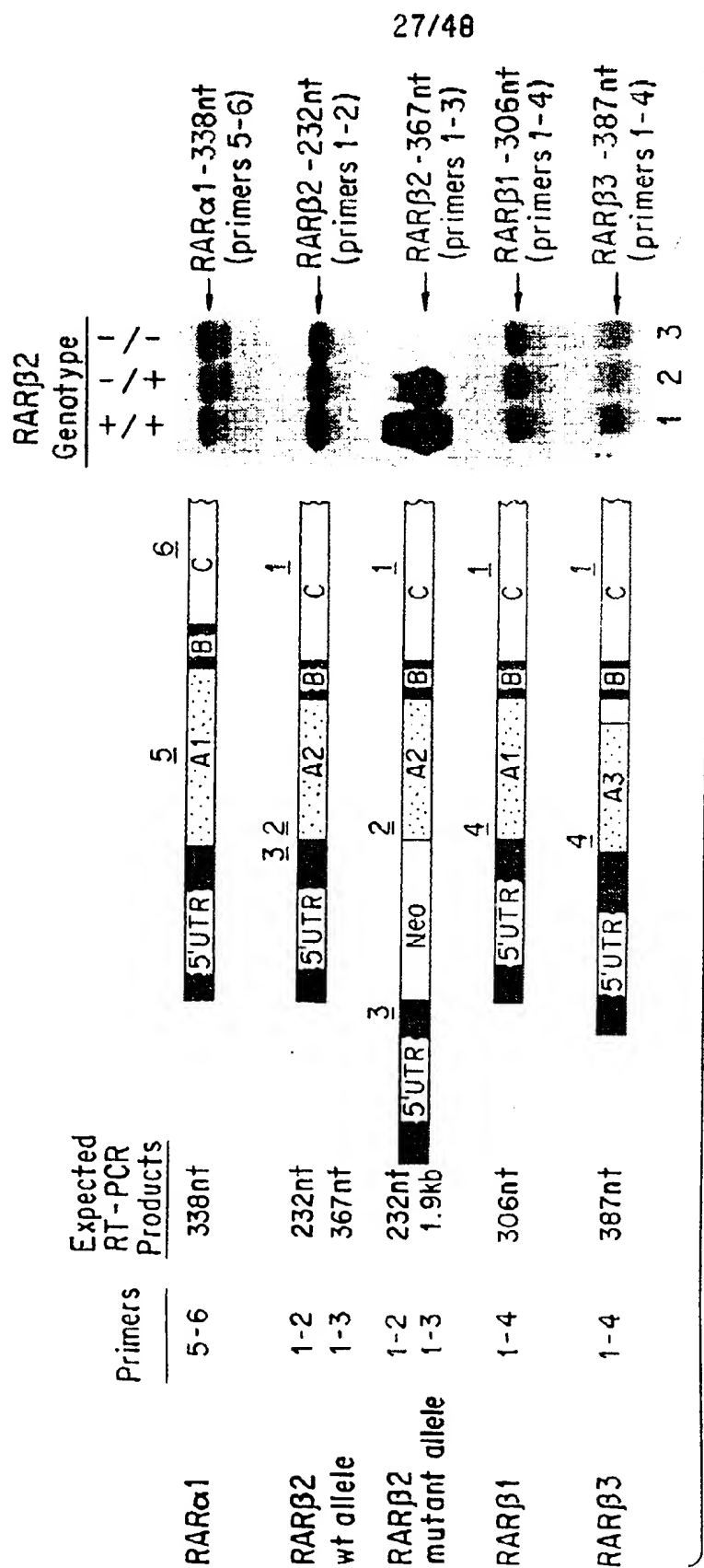


FIGURE 12A

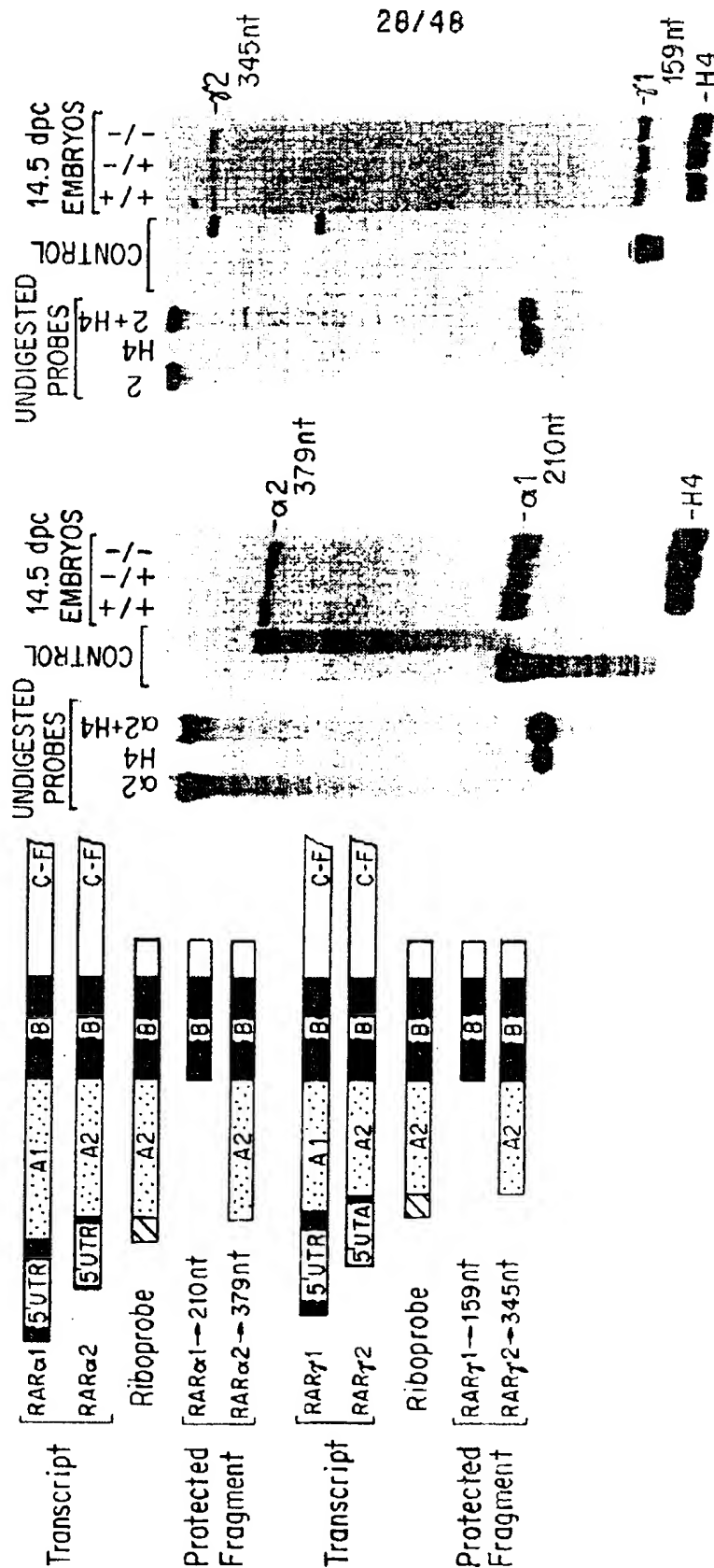


FIGURE 12B

29/48

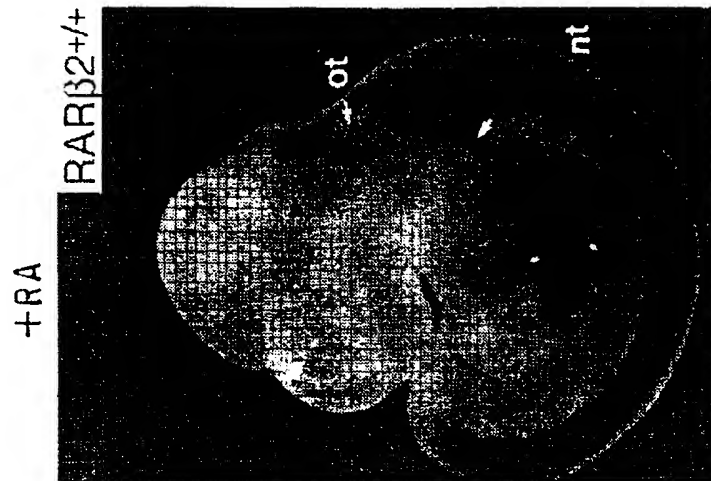


FIGURE 13A

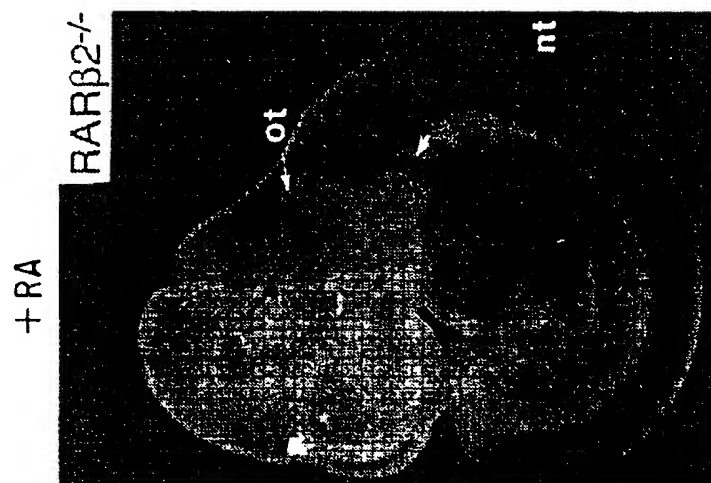


FIGURE 13B

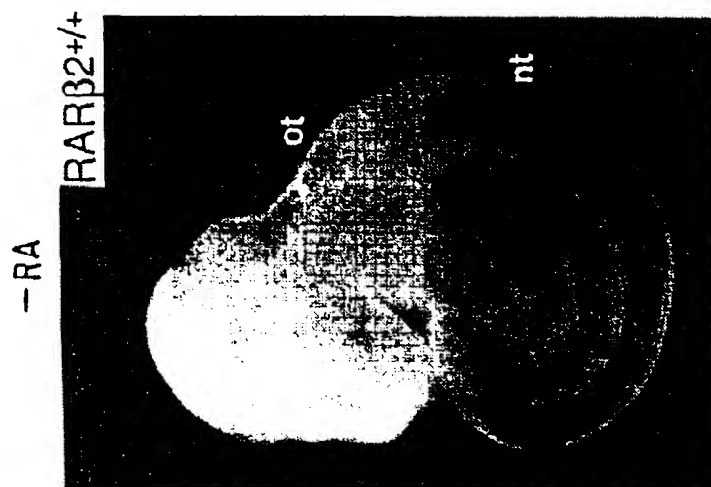


FIGURE 13C



FIGURE 14A

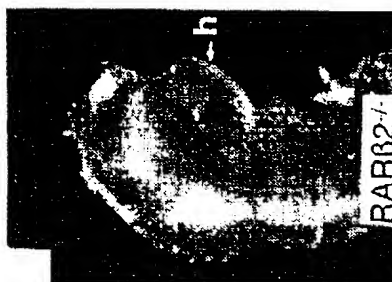


FIGURE 14B

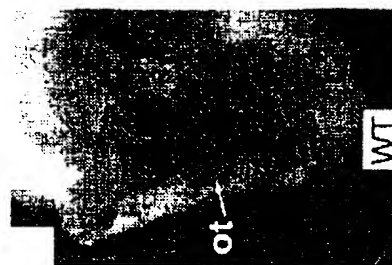


FIGURE 14C



FIGURE 14D

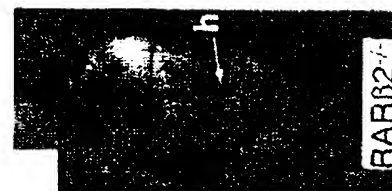


FIGURE 14E

31/48

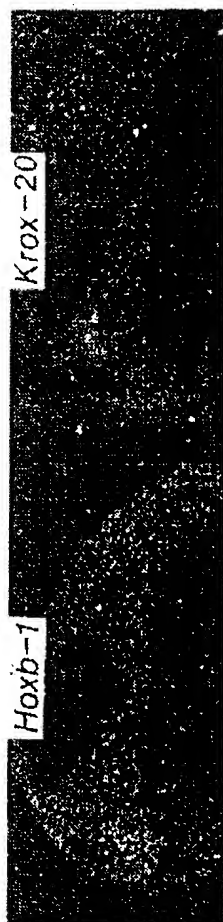


FIGURE 14F

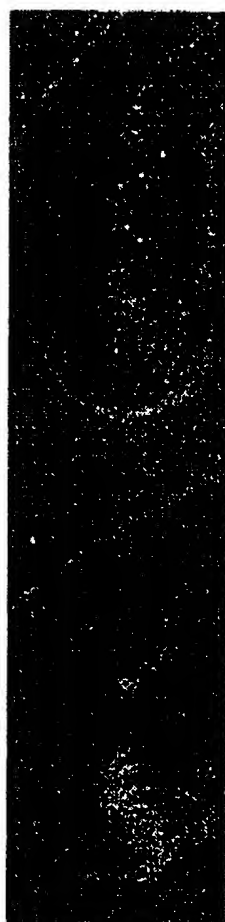


FIGURE 14G

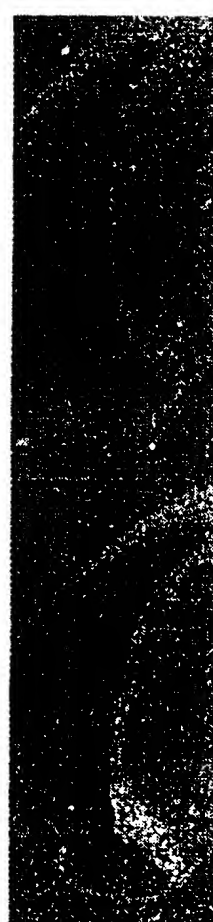
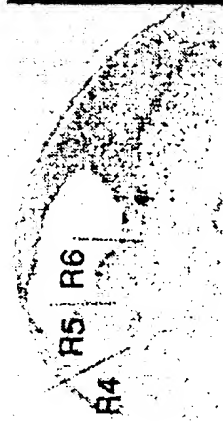


FIGURE 14H



32/48

-RA

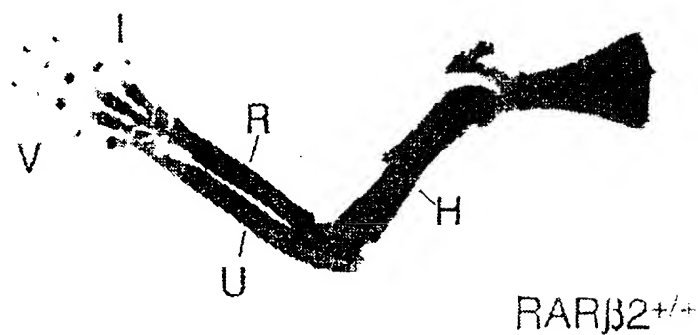


FIGURE 15A

+RA

RAR β 2^{-/-}

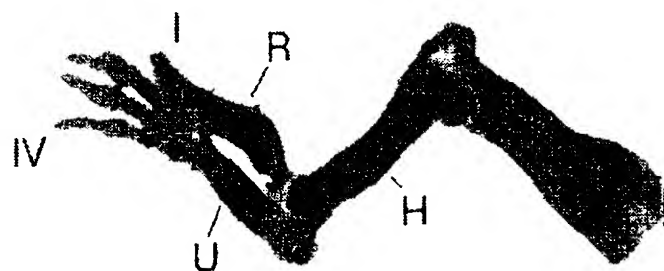


FIGURE 15B

+RA

RAR β 2^{+/+}

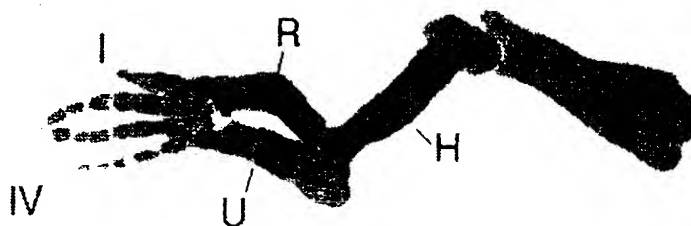


FIGURE 15C

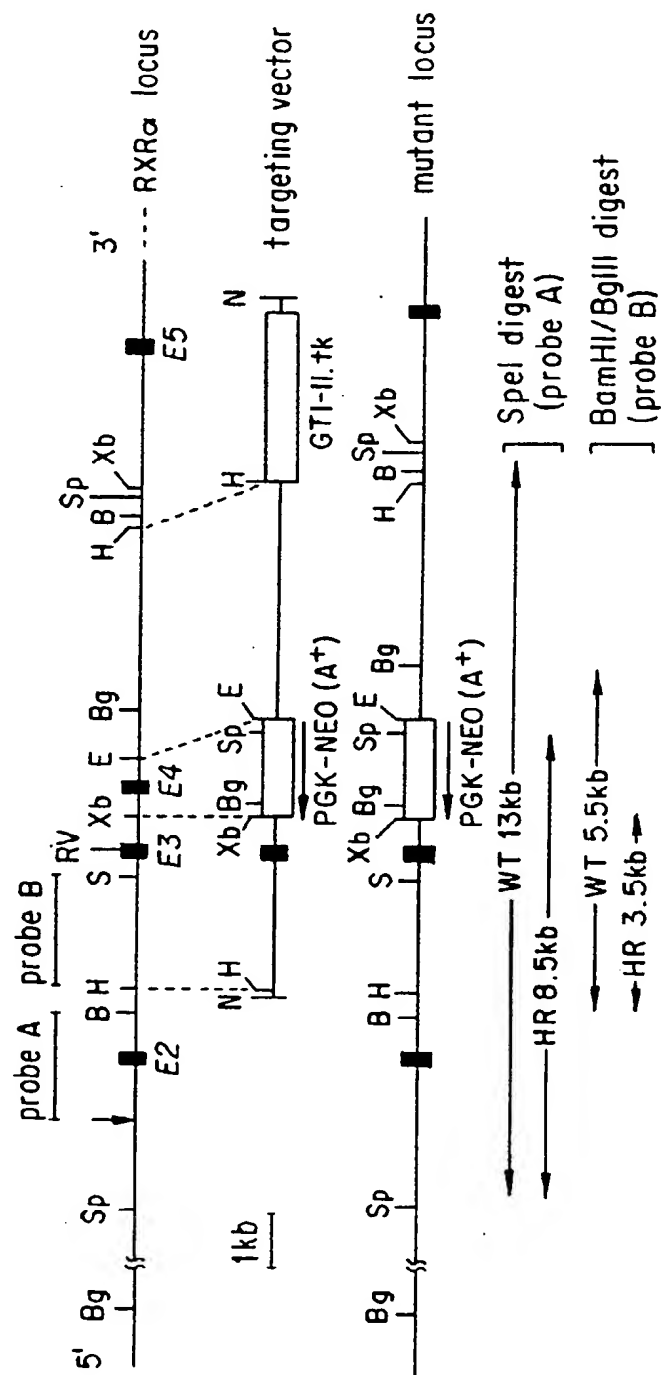
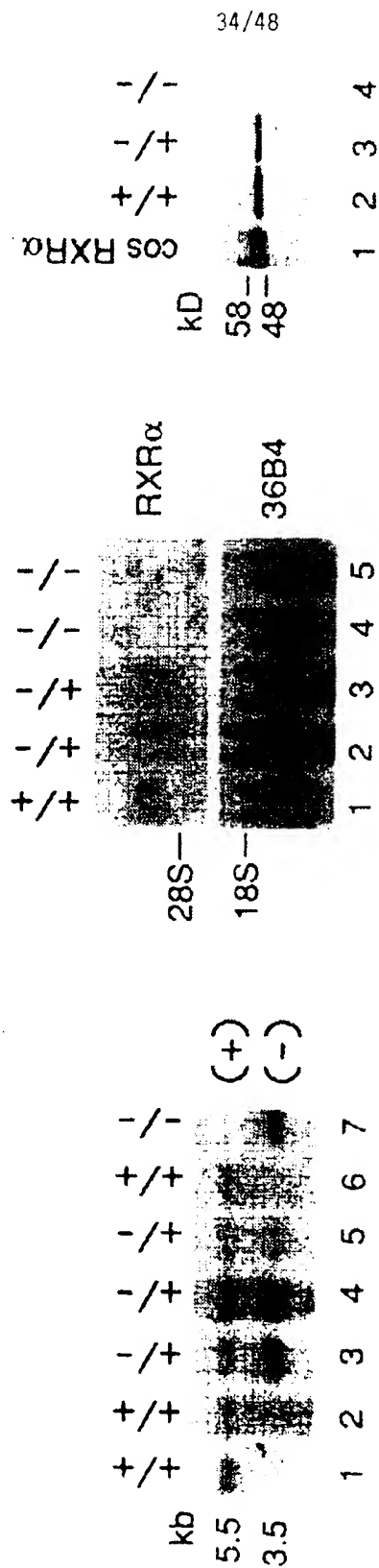


FIGURE 16A



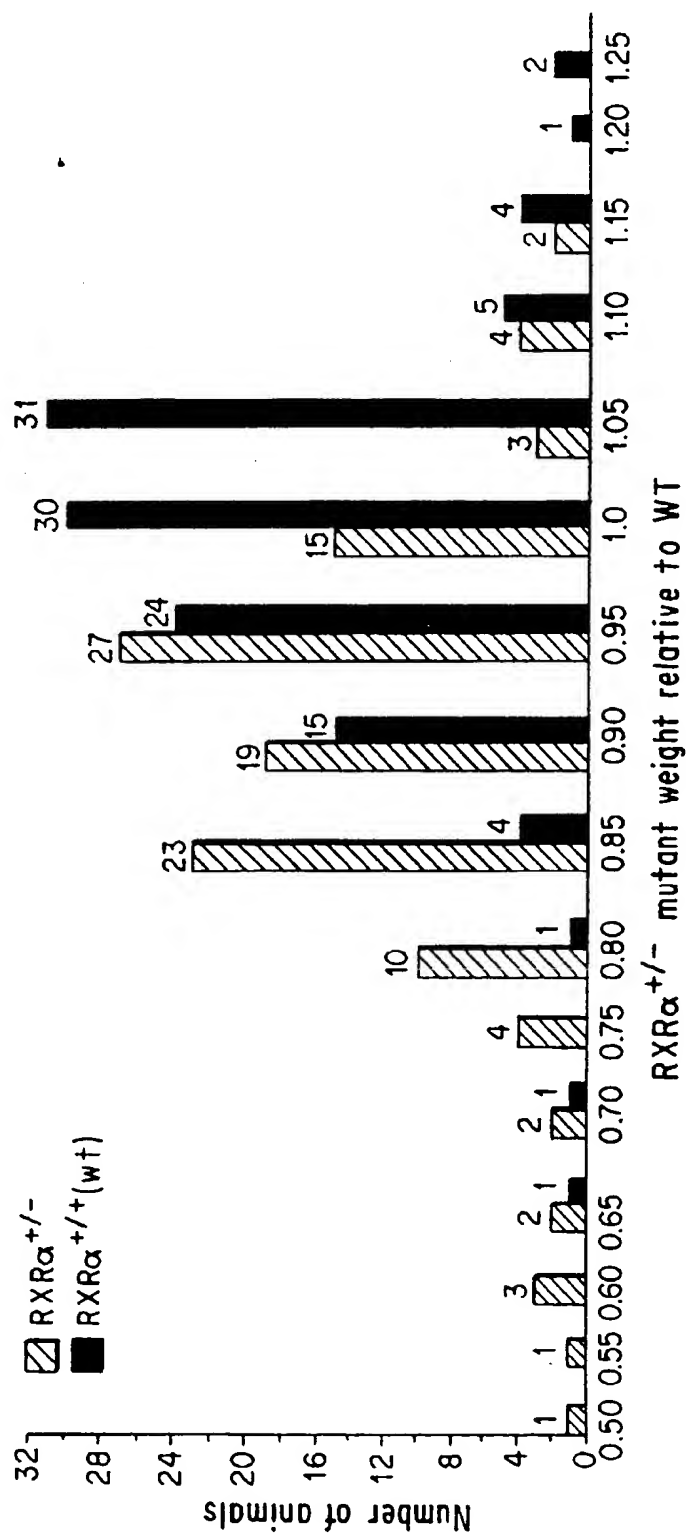


FIGURE 17

36/48



FIGURE 18A



FIGURE 18B

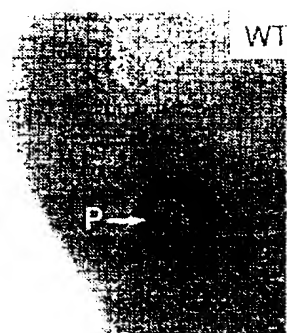


FIGURE 18C

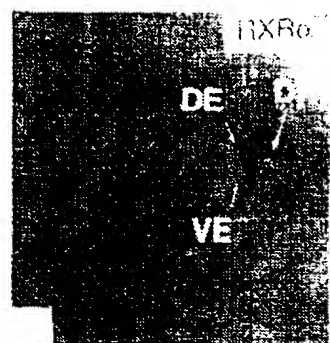


FIGURE 18D

37/48



FIGURE 18E



FIGURE 18F

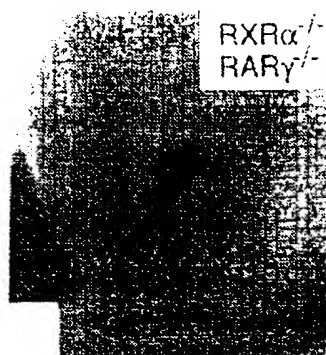


FIGURE 18G

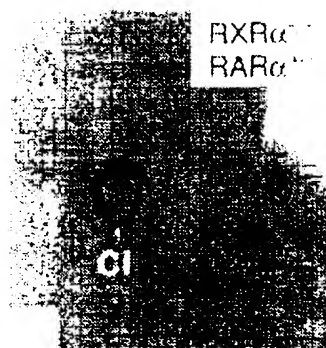


FIGURE 18H

FIGURE 19B



FIGURE 19A



FIGURE 19D



FIGURE 19C



39/48

FIGURE 19E



FIGURE 19F



FIGURE 19G



FIGURE 19H



40/48



FIGURE 20B

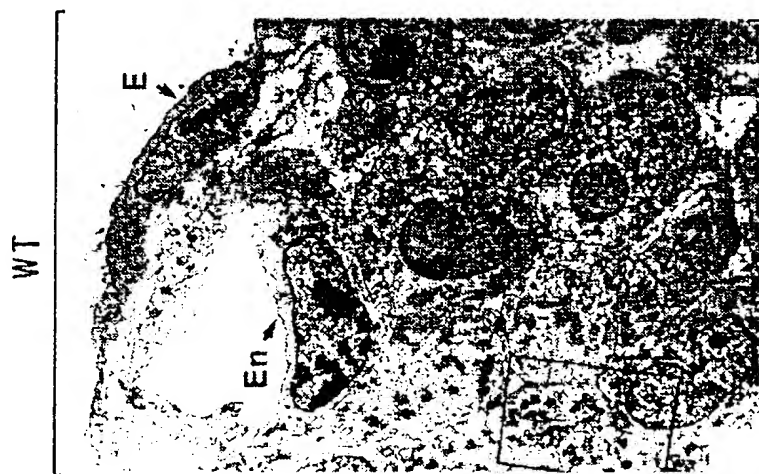


FIGURE 20A



FIGURE 20D

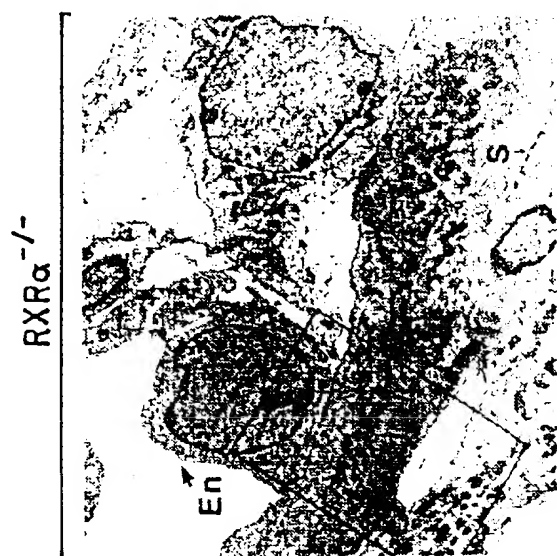


FIGURE 20C

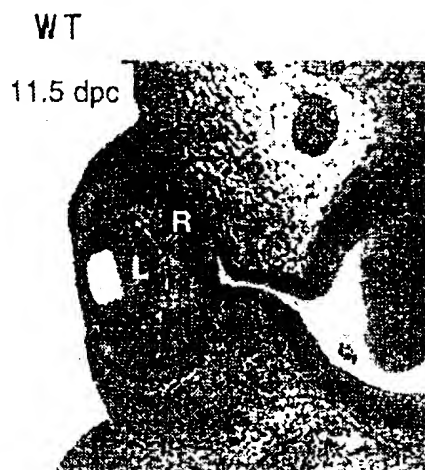


FIGURE 21A

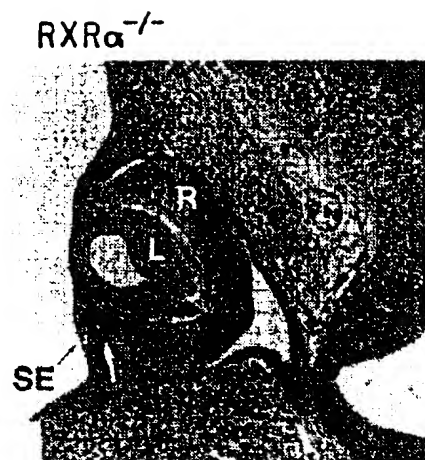


FIGURE 21B

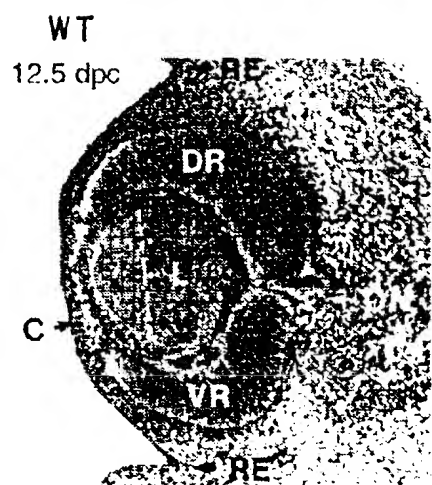


FIGURE 21C

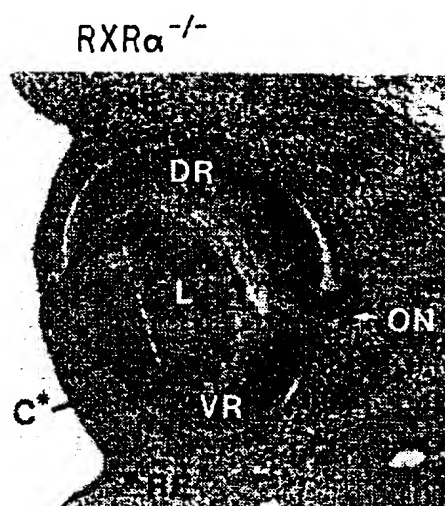


FIGURE 21D

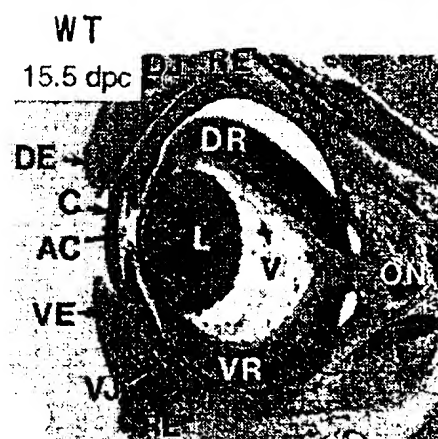


FIGURE 21E

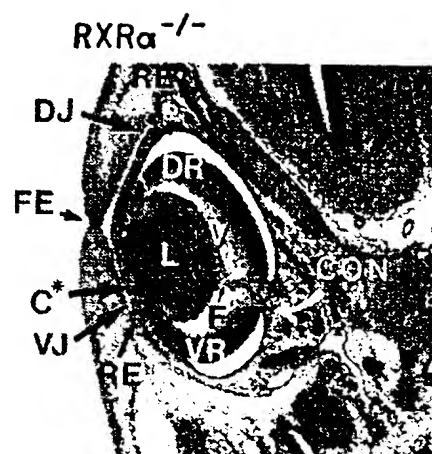


FIGURE 21F

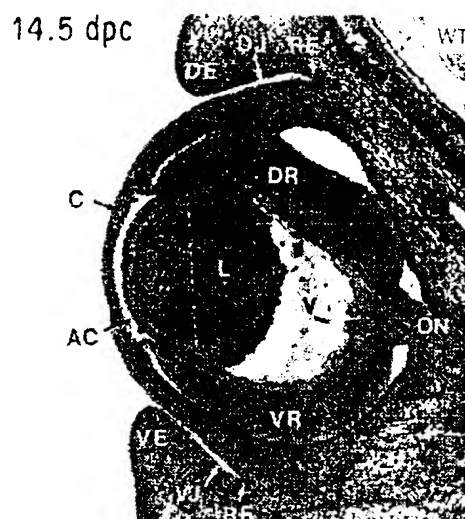


FIGURE 22A

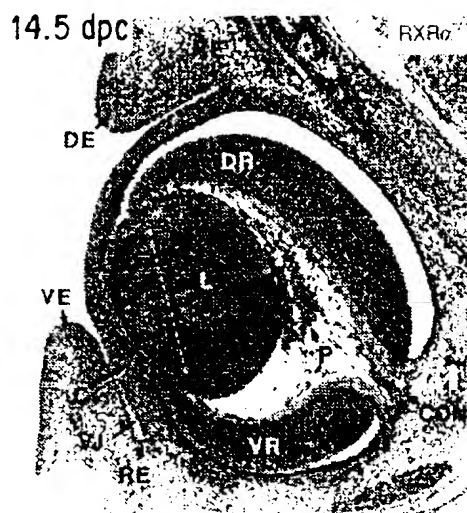


FIGURE 22B

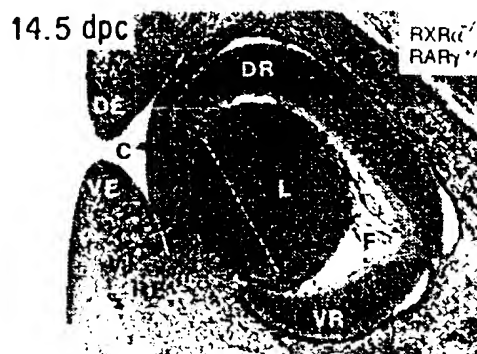


FIGURE 22C

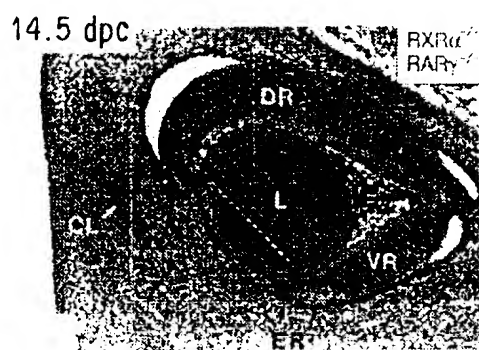


FIGURE 22D

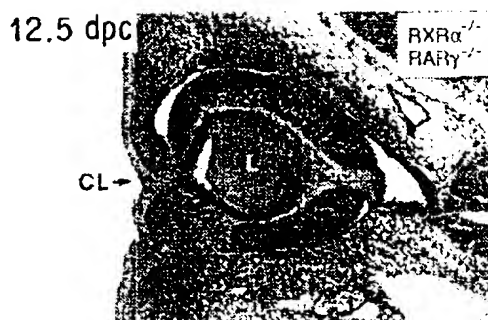


FIGURE 22E

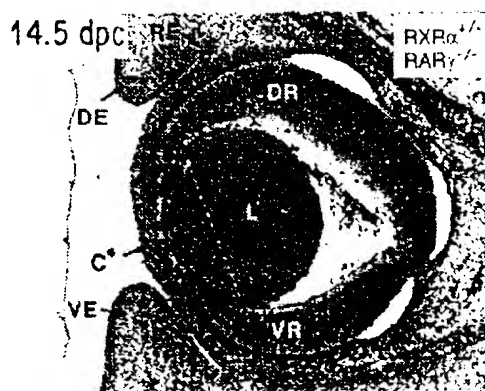


FIGURE 22F

46/48

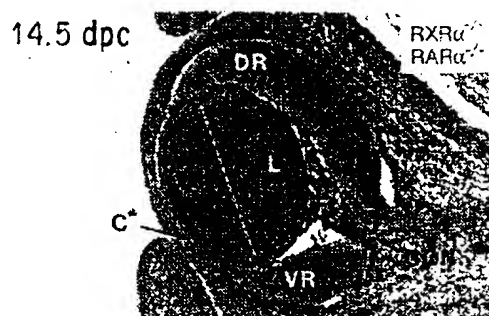


FIGURE 22G

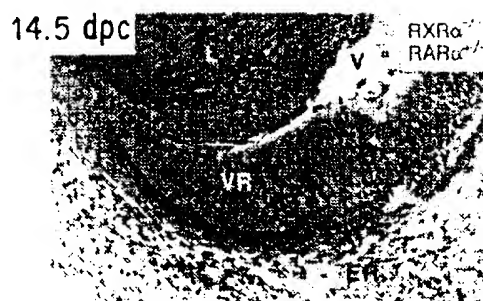


FIGURE 22H

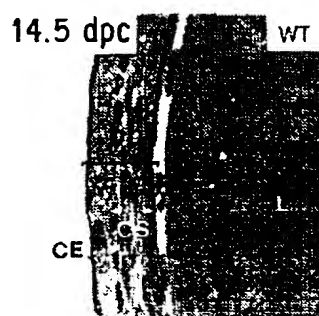


FIGURE 22I

47/48

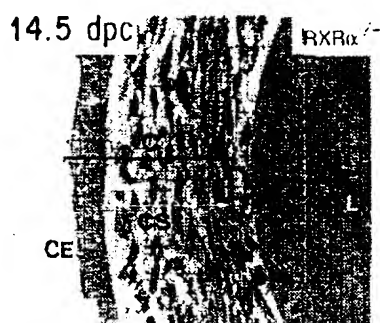


FIGURE 22J

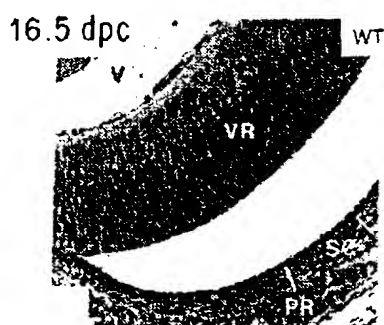


FIGURE 22K

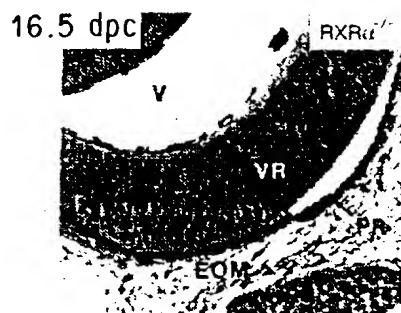


FIGURE 22L

48/48



FIGURE 23A



FIGURE 23B

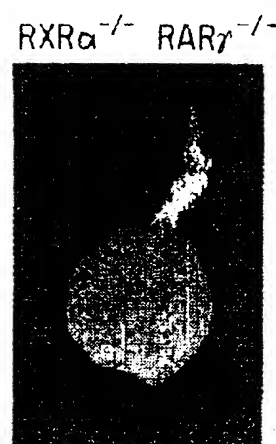


FIGURE 23C

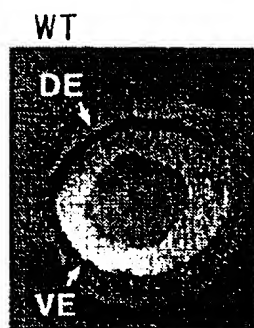


FIGURE 23D



FIGURE 23E

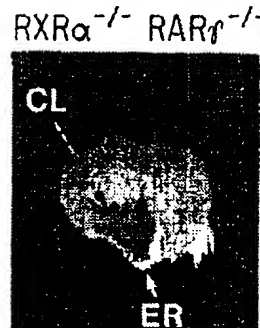


FIGURE 23F



FIGURE 23G



FIGURE 23H

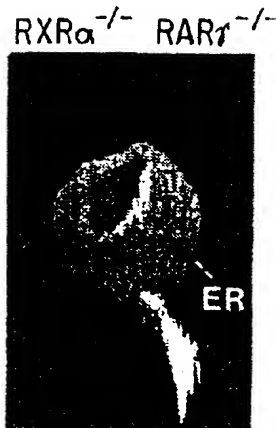


FIGURE 23I

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/05746

A. CLASSIFICATION OF SUBJECT MATTER IPC(5) : A01K 67/00; A61K 49/00; C12N 5/00, 15/00; C12Q 1/00; G01N 31/00, 33/48, 33/53 US CL : 424/9; 435/7.21, 172.1, 172.3, 240.2; 800/2 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 424/9; 435/7.21, 172.1, 172.3, 240.2; 800/2 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) APS, BIOSIS, EMBASE, CHEMICAL ABSTRACTS search terms: transgenic, retinoic acid receptor		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Science, USA, Volume 90, issued January 1993, G. Allenby et al., "Retinoic acid receptors and retinoid X receptors: Interactions with endogenous retinoic acids", pages 30-34, especially pages 30-31.	14, 15, 17, 18
Y	Science, Volume 244, issued 16 June 1989, M.R. Capecchi, "Altering the genome by homologous recombination", pages 1288-1292, see the entire document.	1-18
Y	Genes & Development, Volume 6, issued 1992, D.J. Mangelsdorf et al, "Characterization of three RXR genes that mediate the action of 9-cis retinoic acid", pages 329-344, see the entire document.	1-18
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "Z" document member of the same patent family		
Date of the actual completion of the international search 22 AUGUST 1994		Date of mailing of the international search report 31 AUG 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer BRUCE CAMPBELL <i>B. Kuyza</i> Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/05746

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Development, Volume 113, issued 1991, C. Mendelsohn et al, "Developmental analysis of the retinoic acid-inducible RAR- β 2 promoter in transgenic animals", pages 723-734, see the entire document.	18
X	Genes & Development, Volume 3, issued 1989, A.S. Espeseth et al, "Retinoic acid receptor expression vector inhibits	6, 8
---	differentiation of F9 embryonal carcinoma cells", pages 1647-	-----
Y	1656, see the entire document.	1-5, 7, 9-18
Y	The FASEB Journal, Volume 5, issued November 1991, L.M. De Luca, "Retinoids and their receptors in differentiation, embryogenesis, and neoplasia", pages 2924-2933, especially page 2924.	16
Y	Nature, Volume 339, issued 29 June 1989, A. Zelent et al, "Cloning of murine α and β retinoic acid receptors and a novel receptor γ predominantly expressed in skin", pages 714-717, see the entire document.	1-18